ESCUELA TÉCNICA SUPERIOR
DE INGENIERÍAS AGRARIAS DE PALENCIA

DEPARTAMENTO DE PRODUCCIÓN VEGETAL
Y RECURSOS FORESTALES

**TESIS DOCTORAL**

# CONTROL BIOLÓGICO DEL CHANCRO DEL CASTAÑO MEDIANTE EL EMPLEO DE AISLADOS HIPOVIRULENTOS DEL HONGO *Cryphonectria parasitica* (Murrill) Barr. EN CASTILLA Y LEÓN.

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# Agradecimientos

En primer lugar me gustaría dar las gracias a Julio Diez ya que sin su apoyo y ayuda durante todo el proceso no habría llegado a donde estoy ahora. Es mi "coach" que no me ha permitido abandonar en más de una ocasión además de ser un motor de motivación increíble. También a José Miguel Sierra, Jefe de la Sección de Sanidad Forestal en el momento en el que comencé con este trabajo. Gracias por haber confiado en mí para llevar a cabo todo esto. Gracias por hacerme empezar a trabajar de cero en un laboratorio y darme la oportunidad de aprender todo lo que he aprendido antes y durante este trabajo.

A todo el equipo de Calabazanos que siempre me han apoyado y ayudado, también los que formaron parte del equipo y ahora ya no están. Especialmente a Ana, Eva y Miriam, el "equipo A" del chancro. A Juan Carlos y Gema por sus buenos consejos. A Toni por su ayuda con las técnicas moleculares y por compartir el proceso de desarrollo de una tesis. También a Pablo, Alejandro, Jon y Sara porque juntos hemos aprendido muchas cosas sobre el chancro del castaño.

También quiero dar las gracias a Iñaki, por su apoyo logístico para conseguir artículos además de ser un buen amigo y animarme con su entusiasmo. A Roberto San Martín, gran amigo y gran ayuda con la estadística y porque siempre me hace sentir que el trabajo que hago es interesante.

No puedo olvidar mencionar a Daniel Rigling, Hélène Blauenstein y todo el equipo del centro WSL en Suiza. La estancia en su laboratorio marcó un antes y un después en todo el trabajo. Gracias a ellos aprendí técnicas nuevas que no había empleado antes, desde técnicas moleculares a soluciones muy ingeniosas que facilitan labores muy básicas de laboratorio. Además del trato tan especial que recibí. Fue una experiencia inolvidable.

A Santos, Justi y Miguel por su gran interés en mejorar la situación de los castaños del Bierzo y estar siempre atentos a las novedades que iban saliendo de esta tesis.

Y por último, a mi familia especialmente a Pedro por su paciencia, ánimo y sacrificio. Y a mi hermana Ana y mi amiga Jessica por su gran ayuda con el inglés.

Espero que a todos ellos les guste el resultado.

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# Resumen

La enfermedad del chancro del castaño, producida por el hongo ascomicete C*ryphonectria parastica*, ha diezmado las masas de castaño de Castilla y León desde su primera detección en la región. El control de esta enfermedad se ha llevado a cabo en otros países Europeos así como en España mediante control biológico. Este control consiste en el empleo de aislados de *C. parastica* que contienen un virus conocido como *Cryphonectria hypovirus CHV1* que reduce la virulencia del hongo permitiendo al castaño superar la infección. La transmisión del hipovirus entre dos aislados puede ser horizontal, mediante anastomosis hifal, y vertical, a través de las esporas asexuales. Esta transmisión está condicionada por un sistema de incompatibilidad vegetativa que impide o reduce la transmisión del virus entre aislados que no pertenecen al mismo tipo de compatibilidad vegetativa (tipo de cv, en inglés vc types*, vegetative compatibility types*). La reproducción sexual tiene un efecto negativo en la dispersión del hipovirus ya que las ascosporas nunca contienen hipovirus. Además, la reproducción sexual puede incrementar y mantener la diversidad de tipos de cv a través de recombinación genética. Los objetivos de este trabajo fueron conocer la distribución del chancro en las distintas masas de Castilla y León y estudiar las posibilidades de reducir su impacto a través del control biológico mediante el empleo de aislados hipovirulentos de *C. parasitica*. Tras un intenso muestreo se localizaron las masas afectadas por chancro en León, Zamora, Salamanca y Ávila, provincias donde el castaño aparece en masas relativamente amplias. En estas masas se determinaron los tipos de cv y los tipos de apareamiento (en inglés *Mating types*). En total se determinaron once tipos de cv diferentes en toda la región y la presencia de los dos tipos de apareamiento (Mat-1 y Mat-2) en Zamora y Salamanca, mientras que las masas de León y Ávila solo presentaban Mat-1. Dentro de estos once tipos de cv dos fueron los más frecuentes en la región (EU1y EU11), ambos coincidentes con dos tipos de cv de la colección europea de referencia. Además de conocer la distribución del chancro, se identificaron 15 aislados portadores de virus, el cual se correspondía con el subtipo francés conocido como CHV1-F1. A partir de estos aislados se comprobó la capacidad de transmisión horizontal del hipovirus dentro de aislados del mismo tipo de cv observando que, a diferencia de lo que se ha observado en otros países Europeos, incluso en otras zonas de España, la transmisión horizontal no siempre es del 100% entre aislados del mismo tipo de cv. Los aislados pertenecientes al tipo de cv EU1 presentaron mejores tasas de transmisión que los pertenecientes a EU11. Al analizar los factores que influyen en el crecimiento y la transmisión horizontal y vertical del hipovirus se observó que en el desarrollo de las colonias existe una interacción entre el tipo de cv, el tipo de apareamiento del hongo y el hipovirus que está parasitando al hongo. En la transmisión horizontal solo se observó una influencia del tipo de cv del hongo. Sin embargo, la transmisión vertical a las esporas está influenciada por el virus que coloniza al hongo. En Castilla y León fue más efectivo en cuanto a producción de conidios el hipovirus CHV1-F frente al CHV1-I, al contrario de lo obtenido en otros estudios. Las inoculaciones en campo se llevaron a cabo en León y Zamora. En León estas inoculaciones dieron buen resultado reduciendo el incremento de las lesiones de chancro tanto del tipo de cv EU1 como EU11 al cabo de un año. En cambio en Zamora, sólo uno de los tres tratamientos aplicados de EU11, redujo desarrollo de los chancros inoculados al cabo de un año. En Castilla y León la enfermedad del chancro del castaño se puede controlar en estos momentos mediante el empleo de hipovirulencia. Por ello sería necesaria una dedicada actuación y mayor estudio en las provincias donde aún no está presente el hypovirus, evitando con ello la expansión de la enfermedad y el aumento de la diversidad que pudiera afectar su tratamiento en un futuro.

# Abstract

The chestnut blight produced by the fungus *Cryphonectria parasitica*, has decimated the chestnut stands of Castilla y León since its first discovery in the region. In Europe and Spain this pathogen has been controlled by a biological method. This method consists in using *C. parasitica* isolates infected with a virus known as *Cryphonectria hypovirus* CHV1 that reduces the virulence of the fungus allowing chestnut trees to overcome the disease. The transmission of the hypovirus between two isolates can be horizontal, via hyphal anastomosis, or vertical, through asexual pycnospores. The hypovirus transmission is controlled by a vegetative incompatibility system, that restricts the virus transmission between isolates belonging to different vegetative compatibility types (vc types). The sexual reproduction has a negative effect on the hypovirus spread, because ascospores are always virus free. Moreover the sexual reproduction can increase and maintain vc types diversity through recombination. The aims of this work were to know the chestnut blight distribution in the chestnut stands within Castilla y León and to study the possibility to reduce the impact of the disease through biological control with hypovirulent isolates of *C. parasitica*. After an intensive sampling, the disease was found to occur in chestnut stands of León, Zamora, Salamanca and Ávila, the four provinces where chestnut stands are noteworthy. In these stands we determined the vc type and the mating type distribution, with a total of eleven different vc types in the whole region. We also determined the presence of both mating types (Mat-1 and Mat-2) in Zamora and Salamanca and only Mat-1 in León and Ávila. Two of the eleven vc types were the most frequent in the region and both were coincident with vc types of the European reference collection (EU1 and EU11). Besides the determination of the vc types distribution, 15 hypovirulent isolates were identified, all corresponding to the French subtype known as CHV1-F1. With this isolates we tested the capability of transmission of the hypovirus between isolates from the same vc type and we observed that the hypovirus transmission was not of 100% as in other European countries or other Spanish regions. Isolates from vc type EU1 had better transmission rates than isolates from EU11. During the analysis of factors influencing on the growth and horizontal and vertical transmission of the virus we observed an interaction between vc type, mating type and the hypovirus in the growth of the colonies. The horizontal transmission of the hypovirus was only affected by the vc type of the fungal isolate. On the other hand, the factor influencing the vertical transmission of the hypovirus was the hypovirus parasitizing the fungal isolate. With isolates of *C. parasitica* from Castilla y León, the spore production was greater when the fungal isolate was parasitized with CHV1-F1 than when the hypovirus was CHV1-I, in contrast to other studies. Field inoculations were done in chestnut stands within León and Zamora provinces. In León the hypovirulent inoculations showed good results, reducing the canker lesions from vc type EU1 and EU11 after one year. In contrast, in Zamora, only one of the three treatments used of vc type EU11 had good results reducing canker lesions after one year. In Castilla y León, the chestnut blight disease can be controlled in this moment by biological control with hypovirulent isolates of *C. parasitica*. Hereby a good performance is necessary and more studies in those provinces where the hypovirus is still not present, avoiding the disease expansion and the increase of vc types that could affect a future control with hypovirulence.

# Listado de artículos originales

*Esta tesis está basada en los siguientes artículos originales, a los que se hará referencia en el texto con su correspondiente número (I-IV).*

**I.-** Zamora, P.; Martín, A. B.; Rigling, D.; Diez, J. J. 2012. Diversity of *Cryphonectria parasitica* in western Spain and identification of hypovirus-infected isolates. Forest Pathology 42:412-419.

**II.-** Zamora, P; Martín, A. B.; Arrate, J.; Rigling, D.; Diez, J. J. 2008. Detection of the vegetative compatibility groups of *Cryphonectria parasitica* in Castilla y León region. Acta Hortic. 784:159-162.

**III.-** Zamora, P.; Rigling, D.; Diez, J. J. 2008. Detection of hypovirulent isolates of *Cryphonectria parasitica* in Castilla y León, Spain. Acta Hortic. 784: 163-168.

**IV.-** Zamora, P.; Martín, A. B.; Dueñas, M.; San Martín, R.; Diez, J. J. 2015. *Cryphonectria parasitica* isolates of the same vegetative compatibility type display different rates of transfer of CHV1 hypovirus**.** Eur. J. Plant Pathol. DOI: 10.1007/s10658-015-0727-3

**V.-** Zamora, P.; González-Casas, A.; Martín, A. B.; Dueñas, M.; San Martín, R.; Diez, J. J. 2015. Factors influencing growth, sporulation and virus transfer of *Cryphonectria parasitica* isolates from Castilla y León (Spain). Enviado a European Journal of Plant Pathology

**VI.-** Zamora, P.; Martín, A. B.; San Martín, R.; Martínez-Álvarez, P., Diez, J. J. 2014. Control of chestnut blight by the use of hypovirulent strains of the fungus *Cryphonectria parasitica* in northwestern Spain. Biological Control 79:58-66.

# Introducción

La enfermedad conocida como chancro del castaño fue descubierta por primera vez en Nueva York en 1904. Esta enfermedad supuso el comienzo de un desastre ecológico en las masas forestales de castaño americano (*Castanea dentata* Borkh.). La epidemia fue provocada por el hongo ascomycete denominado *Cryphonectria parasitica* (Murr.) Barr. (Syn. *Endothia parasitica* (Murr.) And. & And.), el cual fue introducido accidentalmente en castaños importados del este asiático. La enfermedad se identificó por primera vez en Italia en castaños europeos (*Castanea sativa* Mill) en 1938 y a partir de este momento se extendió rápidamente a la mayoría de países del sur de Europa (Milgroom y Cortesi, 2004). Actualmente *Cryphonectria parasitica* se encuentra distribuida en numerosos países del continente europeo (Robin y Heiniger, 2001). En España se detectó por primera vez *Endothia parasitica* en Vizcaya en 1947 (Elorrieta, 1949) y en la actualidad este hongo afecta gravemente a las masas de castaños del norte de la Península Ibérica (Homs et al., 2001).

*Cryphonectria parasitica* es uno de los patógenos forestales más agresivos; una vez infectado el árbol este sufrirá un decaimiento progresivo y probablemente morirá (Cobos, 1989). El hongo penetra en el árbol a través de lesiones en la corteza de ramas y tronco, apareciendo como primer síntoma apreciable de la enfermedad un marchitamiento que se produce en ciertas partes de la copa por la pérdida de agua en las hojas de los ramillos terminales. A medida que el patógeno coloniza nuevos tejidos las hojas comienzan a caer y aparecen los típicos síntomas de puntisecado. En los árboles infectados por *C. parasitica* aparecen áreas rojo-anaranjadas en ciertas zonas de la corteza de las ramas y tronco, que se originan como consecuencia de la desecación de los tejidos infectados por el hongo. Estas zonas, ligeramente deprimidas, terminarán arrugándose y agrietándose constituyendo el típico síntoma de chancro que da nombre a la enfermedad. En estas áreas suelen aparecer unas pústulas amarillas o anaranjadas donde se sitúan los cuerpos de fructificación asexual (picnidios) y sexual (peritecios) de este hongo (Figura 1) (Griffin, 1986; Heiniger y Rigling, 1994). Las esporas son arrastradas por el viento, lluvia o adheridas a las patas de pájaros e insectos estableciendo nuevas colonias y difundiendo las ya presentes. Además, este hongo subsiste como parásito benigno sobre otras especies o como un saprófito sobre materia orgánica muerta (Newhouse, 1990).

**Figura 1.-** Cuerpos de fructificación sobre la corteza de un castaño.

Aunque el principal hospedante de *C. parasitica* es el castaño europeo (*Castanea sativa*) (Figura 2), también subsiste como parásito secundario sobre otras fagáceas (Heiniger y Rigling, 1994, Adamčíková et al., 2010). En diversas zonas del mundo, esta especie es apreciada por su madera noble y con gran cantidad de taninos los cuales le confieren la resistencia a la pudrición (Newhouse, 1990). En países como Italia, Francia, Suiza, Portugal y España, cabe destacar el importante papel que juega la producción de castañas en la economía de las poblaciones rurales (Zamora et al., 2012).

**Figura 2.-** Castaño infectado por *Cryphonectria parasitica*.

La resistencia al patógeno de las principales especies de castaño es la siguiente: *C. crenata* y *C. mollisima* son muy resistentes (Heiniger y Rigling, 1994); *C. sequini*, *C. pumila*, *C. ozarkensis* presentan una susceptibilidad intermedia; el *C. henryi* es sensible, mientras que *C. sativa* y *C. dentata* son muy sensibles (Lanier, 1978; Cobos, 1989). No obstante se ha comprobado que todas las especies poseen mecanismos de inmunidad activa que se oponen al ataque del hongo, siendo poco o nada efectivos en las especies susceptibles (Cobos, 1989).

En la actualidad el castaño europeo se distribuye por el área circunmediterránea. En nuestro país puede encontrarse desde el nivel del mar en la Cornisa Cantábrica, hasta los 1500 metros en las montañas andaluzas (López, 1995).

En Castilla y León las masas de castaño puras suponen entorno a las 17.126 ha, según el II Inventario Forestal Nacional, aunque esta cifra puede fluctuar por el efecto de los incendios, abandono de cultivos, enfermedades como el chancro o la tinta así como por las nuevas plantaciones para producción de fruto (Aguín et al., 2005). Las masas de castaño en la región están ampliamente distribuidas en las provincias de León, Salamanca, Zamora y Ávila. La producción de castañas en Castilla y León supone el 25% de la producción total española que es de 40.000 toneladas anuales. León produce aproximadamente, unas 8.000 toneladas, Zamora 1.000 y Salamanca y Ávila 500 toneladas cada una (CESEFOR 2009). Estas masas son principalmente productoras de

fruto (Figura 3), pero la presencia del chancro del castaño ha desanimado a los productores ya que ven mermadas sus producciones de castaña.

**Figura 3.-** Masas de castaño en el paraje de Las Médulas (León).

Un método de control que se ha mostrado efectivo en algunos países como Italia, Francia y Suiza es el control biológico con cepas fúngicas hipovirulentas de *C. parasitica* (Heiniger y Rigling, 1994; Melzer et al., 1997; Bissegger et al., 1997; Cortesi et al., 1998; Anagnostakis et al., 1998). Estos hongos hipovirulentos son individuos que han sido infectados naturalmente en el monte por un micovirus (virus específico de hongos) con un ARN de doble cadena (dsRNA), que les ocasiona una pérdida en su virulencia (Peever et al., 1998; Gobbin et al., 2003). Por ello, pese a que las cepas de chancro hipovirulentas son capaces de infectar a los castaños, no provocan en ellos daños de gravedad permitiéndoles su recuperación en pocos años (Trestic et al., 2001). Los virus más significativos para el control biológico del chancro del castaño son aquellos pertenecientes a la familia *Hypoviridae*, de la que se han descrito cuatro especies (Milgroom y Cortesi, 2004): *Cryphonectria hypovirus* (CHV1), (CHV2), CHV3) y (CHV4), de los cuales CHV1 es el que mayor atención ha recibido por su papel en el control biológico en Europa, mientras que las tres especies restantes han sido detectadas casi exclusivamente en Norteamérica (Alleman et al.,1999; MIilgroom y Cortesi, 2004). La diseminación de estos virus dentro de la población fúngica puede llevarse a cabo mediante esporas asexuales (conidios) infectados o bien mediante el contacto de individuos fúngicos y fusión celular (anastomosis hifal), ya que estos virus se localizan en el citoplasma del hongo (Van Alfen et al., 1975; Hoegger et al., 2003) y en la unión de sus células hifales se produce la transmisión de material citoplasmático de una colonia fúngica a otra.

Sin embargo, la diseminación de la hipovirulencia se ve limitada por dos factores. Por un lado, el mecanismo de transmisión horizontal (anastomosis hifal) está limitado

por un sistema de incompatibilidad vegetativa (vic) del hongo (Homs et al., 2001; Hoegger et al., 2003). Este sistema de incompatibilidad vegetativa es un proceso de regulación genética que suele producir la muerte de las células hifales fusionadas en caso de no ser compatibles (Glass y Kaneko, 2003), aunque se conocen algunos casos en los que se ha producido cierta transmisión entre individuos incompatibles (Cortesi et al., 2001). Por otro lado, no se produce transmisión del virus durante la reproducción sexual de manera que las ascosporas (esporas sexuales), libres de virus (Anagnostakis, 1988; Homs et al., 2001; Gobbin et al., 2003), darán lugar a nuevos chancros virulentos. Además, la reproducción sexual en *C. parasitica* puede incrementar el número de tipos de cv a través de la recombinación de los genes vic polimórficos (Cortesi y Milgroom 1998). Al igual que otros ascomicetes, *C. parasitica* tiene dos alelos de tipos de apareamiento (Mat-1 y Mat-2) en un único locus que controla la compatibilidad sexual (Marra y Milgroom, 2001).

Estos mecanismos de barrera, que impiden la diseminación de la hipovirulencia entre cepas de distintos tipos de compatibilidad vegetativa (tipos de cv), hacen que cuanto mayor sea el número de tipos de cv en una población tanto peor será la diseminación de un hipovirus dentro de la misma (Liu y Milgroom, 1996; Rigling y Henigger, 1999). La alta diversidad de tipos de cv ha hecho que los intentos de diseminación de la hipovirulencia en Norteamérica no hayan sido tan satisfactorios como los progresos obtenidos en distintos países europeos donde la diversidad de tipos de cv es menor (Rigling y Henigger, 1999; Robbins y Griffin, 1999). Por ello, si se quiere conocer la evolución de un castañar afectado por chancro, es necesario conocer previamente los tipos de compatibilidad vegetativa de las poblaciones de *C. parasitica* (actualmente hay más de 74 grupos descritos), ya que de ello depende el mantenimiento y dispersión eficaz de la hipovirulencia (Liu y Milgroom, 1996; Cortesi et al., 1998; Robin et al., 2000; Aguín et al., 2005).

# Objetivos

El chancro del castaño es una enfermedad que provoca importantes pérdidas económicas ya que se reduce la producción de fruto y termina por secar los castaños infectados. Por este motivo, los objetivos generales de esta tesis fueron determinar la distribución del hongo *Cryphonectria parasitica* en las principales masas de Castilla y León y determinar si cabe la posibilidad de controlar la enfermedad mediante el empleo de aislados hipovirulentos como control biológico. Estos objetivos generales se concretaron en:

1.- Conocer la distribución del chancro en las principales masas de castaño (I)

2.- Determinar los diferentes tipos de compatibilidad vegetativa (tipos de cv) de la región (I, II)

3.- Analizar la distribución de los tipos de apareamiento (mating types) existentes en las masas de Catilla y León (I).

4.- Comprobar la existencia o no de aislados hipovirulentos en las masas de castaño (I, III).

5.- Analizar la capacidad de conversión de los aislados hipovirulentos de Castilla y León (IV).

6.- Determinar qué factores influyen en la transmisión horizontal y vertical del hipovirus entre aislados del mismo tipo de compatibilidad vegetativa (V).

7.- Determinar el efecto del control biológico mediante la inoculación de castaños afectados por chancro con aislados hipovirulentos (VI).

# Materiales y Métodos

*Muestreo (I, II)*

El muestreo se llevó a cabo en 44 masas afectadas de castaño distribuidas en las cuatro provincias de Castilla y León donde las masas de castaño son abundantes: León, Zamora, Salamanca y Ávila.

*Aislamiento de Cryphonectria parasitica (I, II)*

A partir de las muestras de corteza obtenidas de los chancros muestreados, tras una desinfección superficial se sembraron en medio de cultivo no selectivo compuesto por patata, agar y dextrosa (PDA)

*Identificación de los tipos de compatibilidad vegetativa (tipos de cv) (I, II)*

Los tipos de cv se determinaron mediante la respuesta de barrera/fusión (Anagnostakis et al., 1986). El medio utilizado en este caso fue PDA modificado (Powell 1995). Las confrontaciones se realizaron entre aislados de Castilla y León y de éstos con los aislados EU1-EU74 de la colección europea de referencia (Cortesi y Milgroom, 1998; Robin et al., 2000)

*Análisis de los tipos de apareamiento (mating types) (I)*

Los tipos de apareamiento se determinaron para una submuestra de 591 aislados elegidos al azar. El análisis se llevó a cabo mediante una amplificación PCR con los iniciadores M1-GS1 y M1-GS2-rev para MAT-1 y M2-GS2 e InvA5n para MAT-2 (Marra y Milgroom, 1999; McGuire et al., 2001).

*Identificación de aislados hipovirulentos y análisis de la secuencia de los hipovirus (I, III, IV)*

La identificación de los aislados hipovirulentos de *C. parasitica* se basó en el análisis morfológico (observación de la pigmentación y esporulación) y molecular (extracción de dobles cadenas de ARN, dsRNA) (Morris y Dodds 1979; Rigling et al. 1989). Los hipovirus encontrados se compararon con los subtipos conocidos del hipovirus CHV1 (Gobbin et al. 2003) mediante la secuenciación de una región específica del ARN del virus.

*Análisis de la conversión entre aislados donantes y receptores de C. parasitica de los principales tipos de cv de Castilla y León (IV, V)*

La capacidad de transmisión del hypovirus se determinó mediante el método de enfrentamiento de parejas en medio de cultivo no selectivo (PDA) (Anagnostakis y Day, 1979; Liu y Milgroom, 1996; Cortesi et al., 2001; Papazova-Anakieva et al., 2008). Para comprobar el éxito de conversión se realizó posteriormente la extracción de dsRNA.

*Crecimiento de aislados virulentos e hipovirulentos a 15 y 25ºC (V).*

Los aislados se cultivaron en medio no selectivo (PDA) a 15ºC y 25ºC y se midieron dos ejes perpendiculares durante siete días.

*Análisis de la tasa de producción de conidios, caracterización de cultivos monospóricos (V).*

La producción de conidios se llevó a cabo sometiendo a las colonias a condiciones de luz forzada (2500 lux) durante 10 días con fotoperiodo de 14 h de luz. Los cultivos monospóricos se obtuvieron sembrando una espora por placa de medio de cultivo no selectivo PDA.

*Preparación de inóculo hipovirulento (VI)*

Los aislados hipovirulentos seleccionados se cultivaron en medio de cultivo no selectivo PDA. Las colonias obtenidas se trituraron en condiciones de esterilidad y se entubaron en tubos de aluminio conservándose a 4ºC hasta su empleo en las inoculaciones.

*Inoculación de estacas en laboratorio (VI)*

Las estacas empleadas para las inoculaciones en laboratorio fueron de *Castanea sativa* de 130 cm de longitud y 3-5 cm de diámetro. En total se emplearon 65 estacas de castaño. El área de las lesiones se estimó mediante la fórmula de la elipse (Elliston, 1978; Turchetti y Maressi, 1991).

*Inoculación en castaños con chancro en campo (VI)*

Las inoculaciones se llevaron a cabo en Médulas y Berlanga del Bierzo en León y Robledo en Zamora. En total se inocularon 9 hectáreas (54 pies) en Médulas, 6 ha (35 pies) en Berlanga del Bierzo y 4 ha (25 pies) en Robledo.

*Análisis estadísticos (I, II, III, IV, V, VI)*

Indice de Shannon y Wiener (I, II).

Modelos mixtos para determinar: La capacidad de conversión (IV, V), producción de conidios (V), efecto del hipovirus sobre el crecimiento (VI)

Modelo de medidas repetidas para calcular el efecto de las inoculaciones en campo (VI) con contraste ortogonales y test LSD para determinar las diferencias significativas.

Mediante un Análisis de Correspondencias simples (ACS) y un análisis Clúster se caracterizaron aislados monospóricos virulentos e hipovirulentos (V)

Todos los análisis realizados se llevaron a cabo mediante el programa informático SAS for Windows 9.2.

# Resultados

**1.- Distribución del chancro en las principales masas de castaño de la región (I).**

El chancro del castaño se encontró en las cuatro provincias de Castilla y León con masas destacables de castaño: León, Zamora, Salamanca y Ávila. En las provincias de León y Zamora su presencia es más abundante en comparación con Salamanca y Ávila. En cuanto a la diversidad, Zamora es la provincia con mayor diversidad de poblaciones de *C. parasitica* y León es la menos diversa.

**2.- Diversidad de tipos de compatibilidad vegetativa (tipos de cv) (I, II)**

Para determinar los diferentes tipos de compatibilidad vegetativa existentes en Castilla y León se analizaron un total de 1.232 aislados. Con estos aislados se lograron obtener un total de 11 tipos de cv en total, cinco con equivalencia en la colección europea (EU-1, EU-11, EU-12, EU-66, EU-28) y seis tipos de cv nuevos no descritos hasta el momento (CL4, CL5 CL6, CL8, CL9 y CL10). El tipo de cv principal fue EU11 seguido de EU1. Los demás tipos de cv fueron mucho menos frecuentes. Atendiendo a su distribución, EU11 apareció en todas las provincias mientras que EU1 solo en tres de las cuatro provincias afectadas por chancro. EU12 y CL4 solo se encontraron en León y Zamora mientras que EU66 y EU28 (con un solo aislado) son exclusivas de Zamora. El tipo de cv denominado CL5 se encontró en Ávila mientras que CL6 principalmente en Salamanca. El tipo CL8 de Zamora solo tenía tres aislados y CL9 y CL10 de Salamanca solo un aislado en cada caso.

**3.- Distribución de tipos de apareamiento (mating types) existentes en Castilla y León (I).**

En cuanto a la distribución de los tipos de apareamiento, en León y Ávila solo se encontraron aislados de MAT-1 excepto un único asilado MAT-2 de León. En Zamora y Salamanca sin embargo había aislados tanto de MAT-1 como de MAT-2. En Salamanca fueron más frecuentes los aislados de MAT-1 y en Zamora los de MAT-2.

**4.- Existencia de aislados hipovirulentos en las masas de castaño (I, III).**

León fue la única provincia que presentó aislados hipovirulentos. En total se encontraron catorce aislados que contenían dsRNA, de los cuales nueve pertenecían al tipo de cv EU1 y cinco a EU11 y todos ellos pertenecían a MAT-1. Todos presentaban morfología hipovirulenta. Doce de los catorce aislados tenían una banda de dsRNA de 12kbp aproximadamente (los dos aislados restantes se perdieron por contaminación). El análisis parcial de la secuencia del genoma del virus reveló un grupo de 9 aislados hipovirulentos con una secuencia idéntica. Tres aislados se distinguían de los anteriores

por dos nucleótidos. El árbol filogenético mostró que los 12 aislados hipovirulentos se agrupaban con el hipovirus CHV1-EP713 que es el hipovirus de referencia para el subtipo francés conocido como F1 y eran claramente distintos del resto de subtipos CHV1.

**5.- Capacidad de conversión de los aislados hipovirulentos de Castilla y León (IV).**

Las conversiones donde los aislados virulentos e hipovirulentos pertenecían al mismo grupo compatible mostraron que aquellos que pertenecían al tipo de cv denominado EU1 presentaban mejores tasas de conversión que los que pertenecían a EU11. Además los aislados obtenidos en las masas de castaño de León se convirtieron mejor que aquellos cuyo origen era Zamora, Salamanca o Ávila. Sin embargo, la interacción entre el factor genotipo del aislado de la misma provincia y tipo de cv fue significativa de manera que los aislados EU1 que mejor conversión presentaron fueron los de León y Zamora seguidos de Salamanca. Mientras que en el caso del tipo de cv EU11 los aislados con una conversión medianamente aceptable fueron los de León y en menor medida los de Ávila, Salamanca y Zamora.

En el caso de las conversiones cruzadas, es decir, cuando los aislados virulentos e hipovirulentos pertenecían a tipos de cv diferentes, la conversión se vio afectada significativamente por el tipo de cv del donante y el receptor y la interacción de ambos. Ninguno de los aislados donantes logró convertir a los aislados pertenecientes a los tipos de cv EU12 y EU66. En cuanto a los receptores CL5 y CL6 se observó que el primero (CL5) solo se convirtió con los donantes CL6 y EU11 mientras que CL6 se convirtió con los donantes de EU1 y peor con los de EU11.

**6.- Factores que influyen en el crecimiento, esporulación y en la transmisión horizontal y vertical del hipovirus entre aislados del mismo tipo de compatibilidad vegetativa (V).**

El crecimiento de los aislados a 25ºC fue mayor que a 15ºC y su comportamiento fue diferente. A 15ºC aquellos aislados que portaban el hipovirus CHV1-I crecieron menos que los que portaban el hipovirus CHV1-F1 y los aislados virulentos crecieron tanto o más que los aislados hipovirulentos en EU1. Pero a 25ºC los aislados virulentos son los que menos crecen. Además, a esta temperatura los aislados del tipo de cv EU1 crecen más que los del tipo EU11. En cuanto a los hipovirus, el que más influyó en el crecimiento de las colonias fue CHV1-I en los aislados de EU1 pero no en los de EU11.

Los aislados virulentos fueron los que más conidios desarrollaron (para ambos tipos de cv, EU1 y EU11) seguidos de los aislados hipovirulentos que portaban el hipovirus CHV1-F1 y por último los que portaban el hipovirus CHV1-I.

Según la caracterización de los aislados basada en características morfológicas como pigmentación (anaranjada o blanca), tipo de micelio (más compacto o algodonoso), esporulación (mayor o menor cantidad de cuerpos de fructificación) y la posterior detección de la presencia o ausencia de virus mediante extracción de ds RNA (RNA de doble cadena), se forman tres clases: clase 1 virulentos, clase 2 intermedios y clase 3 hipovirulentos. La clase virulentos incluyó todos los aislados virulentos y el aislado ZA182 infectado con el hipovirus CHV1 subtipo I. La clase 2 o intermedios solo contó con el aislado ZA478 infectado con ambos subtipos de hipovirus (F1 e I) mientras que la clase 3 o hipovirulentos englobó todos los aislados hipovirulentos menos los mencionados anteriormente.

La conversión solo se vio afectada por el tipo de cv de manera que la transmisión horizontal del hipovirus fue mejor dentro del tipo de cv EU1 que dentro de EU11 para las dos temperaturas ensayadas.

**7.- Efecto del control biológico mediante la inoculación de castaños afectados por chancro con aislados hipovirulentos (VI).**

La inoculación previa en ramas en laboratorio no mostró ninguna diferencia para los aislados del tipo de cv EU1 mientras que en EU11 algunos de los aislados hipovirulentos redujeron la necrosis producida por los aislados virulentos.

En cuanto a la inoculación en campo, a los seis meses de la inoculación no se observaron diferencias significativas entre los chancros inoculados y los chancros control. Sin embargo a los 12/18 meses (según ensayos) los chancros inoculados del tipo de cv EU1 de Berlanga del Bierzo (León) redujeron su crecimiento así como los de EU11 de la inoculación hecha en Médulas (León) mientras que los chancros de EU11 en Robledo (Zamora) solo presentaron una reducción en las necrosis con uno de los inóculos empleados.

# Discusión

**1.- Distribución del chancro en las principales masas de castaño de la región (I).**

La enfermedad producida por el hongo *C. parasitica,* conocida con el nombre de chancro del castaño, se encuentra distribuida en las provincias de León, Zamora, Salamanca y Ávila. La enfermedad se encuentra ampliamente distribuida por León y Zamora mientras que en Salamanca y Ávila se encuentra en expansión.

**2.- Diversidad de tipos de compatibilidad vegetativa (I, II)**

En Castilla y León se determinaron un total de 11 tipos de cv siendo los dos más abundantes en la región EU1 y EU11 seguidos de EU12, EU66, CL6 y CL5. Castilla y León presenta un número relativamente alto de tipos de cv diferentes aunque la mitad de ellos tienen muy pocos aislados (menos de 10). Comparando los resultados de Castilla y León con las regiones colindantes se observó que Galicia tiene 4 tipos de cv (Montenegro et al., 2008) y comparte EU1 y EU66 con Castilla y León. En Cataluña el tipo de cv más extendido es EU2 (Colinas et al. 2009) y EU66 en Navarra-Pyrénée (Robin et al. 2009). Además EU66 se encuentra en Castilla y León, en Portugal y en el noroeste de Francia (Bragança et al. 2007; Robin et al. 2009). Pese a que en Europa es frecuente encontrar como tipos de cv más comunes EU1 y EU2 (Robin and Heiniger 2001), en el caso de Castilla y León parece que solo se ha introducido EU1. Asturias aunque limita la región por el norte, presenta como tipos de cv principales EU3 y EU13 que no están presentes en Castilla y León (González-Varela et al., 2011). En cambio Portugal que comparte frontera con Zamora y Salamanca presenta EU11 como tipo de cv más extendido seguido de EU12 y EU66 (Bragança et al., 2007). EU11 es un tipo de cv raro en Europa encontrándose aparte de Portugal y Castilla y León, en Italia, Francia y Croacia con muy pocos aislados (Cortesi et al. 1996; Robin et al. 2000; Krstin et al. 2008). EU12 es el tipo de cv más extendido en el sureste europeo (Sotirovski et al. 2004; Perlerou and Diamandis 2006; Krstin et al. 2008; Milgroom et al. 2008) y también está presente en la provincia de Zamora. Las masas de castaño de Zamora son las más próximas a Portugal, lo que puede explicar las similitudes de sus poblaciones con las del país vecino. Sin embargo, a diferencia de Castilla y León, en Portugal es raro encontrar aislados pertenecientes a EU1.

En principio se pensó que el chancro del castaño se introdujo desde Francia a España por el transporte de madera y plantas contaminadas (Cobos, 1989) y es probable que EU1 y EU66 hayan seguido esta ruta de entrada hasta Castilla y León. Sin embargo, es posible que EU11 y EU12 en cambio, hayan entrado desde Portugal hacia Zamora y Salamanca y después se hayan extendido hacia León y Ávila.

### 3.- Distribución de los tipos de apareamiento (mating type) (I)

Los aislados de León presentaron MAT-1 (Montenegro et al., 2008) en prácticamente todos sus aislados, al igual que en Ávila, lo que parece indicar que estas poblaciones se han desarrollado a partir de la introducción de tres clones (EU1, EU11 y CL5 todos MAT-1) Esta expansión de tipo clonal también se ha visto en otras partes del sureste de Europa (Milgroom et al. 2008) y norte de Suiza (Hoegger et al. 2000). La dominancia de MAT-1 en estas dos provincias indica que la reproducción es más bien de tipo asexual, lo que hace que la diversidad de tipos de cv también se mantenga baja. Sin embargo en Salamanca y Zamora ambos tipos de apareamiento están presentes lo que hace que tengan mayor reproducción sexual y más diversidad de tipos de cv, lo que puede representar un inconveniente para abordar el control de la enfermedad en un futuro.

### 4.- Existencia de aislados hipovirulentos en las masas de castaño (I, III).

Los aislados de *C. parasitica* que contenían dsRNA en las masas de Castilla y León fueron relativamente escasos ya que tan solo 14 de 1.232 aislados analizados contenían el hipovirus. Estos aislados hipovirulentos se encontraron en las masas de castaño de la provincia de León únicamente. El análisis de la secuencia de dsRNA indicó que todos los aislados contenían el hipovirus CHV1 subtipo francés F1. Estos resultados coinciden con un estudio anterior donde se encontraron 15 aislados en la provincia de León también con CHV1-F1 (Montenegro et al., 2008). Ninguno de los aislados hipovirulentos estaba relacionado con el virus CHV1 subtipo italiano (I) que es el más frecuente en Europa (Allemann et al. 1999; Sotirovski et al. 2006; Krstin et al. 2008; Robin et al. 2010) y en Cataluña (Homs et al. 2001). El hipovirus CHV1-F1 solo se encontró en Francia en el año 1970 (Allemann et al. 1999) por lo que es posible que se haya introducido en León desde Francia a través de material afectado por *C. parasitica* hipovirulento. En cuanto a Portugal, también se han encontrado aislados hipovirulentos (Bragança et al. 2007), pero no se sabe a qué subtipo pertenecen. Sería interesante conocer la distribución de la hipovirulencia en Portugal para averiguar si tiene alguna relación con lo encontrado en Castilla y León, teniendo en cuenta que los tipos de cv son bastante similares. La falta de aislados hipovirulentos en las provincias restantes se puede deber a que la aparición del patógeno es relativamente reciente en algunas masas de la región, como ocurre con las poblaciones de Salamanca y Ávila. En Europa el nivel de hipovirulencia es bajo en las zonas donde *C. parasitica* es reciente (Hoegger et al. 2000; Bragança et al. 2007). Los aislados hipovirulentos encontrados pertenecen a los dos tipos de cv más frecuentes en la región, EU1 y EU11. Estos dos tipos de cv suponen el 88% de los aislados analizados lo que sugiere que el potencial para la dispersión de la hipovirulencia con éxito es alto.

**5.- Capacidad de conversión de los aislados hipovirulentos de Castilla y León (IV).**

La transmisión del hipovirus entre los aislados de *C. parasitica* de Castilla y León fue diferente en función del tipo de cv que se empleó, tanto cuando donante y receptor pertenecían al mismo tipo de cv como cuando pertenecían a dos tipos de cv distintos. Además, la tasa de conversión también varió dependiendo del origen de los aislados (Zamora, Salamanca, León y Ávila) Cuando la conversión se hizo entre aislados del mismo tipo compatible ésta no se produjo en el 100% de los casos como suele ocurrir cuando el aislado donante y receptor pertenecen al mismo tipo de cv (Liu y Milgroom, 1996; Cortesi et al., 2001; Ding et al., 2007; Papazova-Anakieva et al., 2008; Bryner and Rigling, 2012). Aunque la conversión fue en general alta con los aislados del tipo de cv EU1, la conversión con aislados de EU11 fue muy baja variando desde 4 a 58%. No se conocen más casos en los que se produzca una transmisión tan baja del hipovirus cuando el donante y receptor pertenecen al mismo tipo de cv.

La eficiencia en la conversión para EU11 fue mayor con aislados de León que con aislados de Ávila, Salamanca y Zamora. Esto puede deberse al carácter clonal de la población de León que hace que el hipovirus se transmita mejor debido a la falta de diversidad genética (Milgroom et al., 2008; González-Varela et al., 2011).

Otros estudios han demostrado la influencia del hipovirus en la capacidad de conversión (Bryner y Rigling, 2011; Sotirovski et al., 2011) observando que el hipovirus F1 producía las mayores tasas de conversión. Pero esto no concuerda con los resultados obtenidos con los aislados de Castilla y León para el tipo de cv EU11. La influencia del genotipo de los aislados de Castilla y León puede que explique la desigualdad de transmisión del hipovirus.

Cuando la conversión fue con donantes y receptores de distintos tipos de cv, no se produjo transmisión del hipovirus en absoluto a los receptores de EU12 y EU66. Aunque EU12 y EU11 solo son heteroalélicos en el locus vic2, éste es conocido por inhibir fuertemente la transmisión de hipovirus (Cortesi et al., 2001). Sin embargo, cuando los receptores fueron aislados de CL5 y CL6 si se produjo transmisión del hipovirus en algunas ocasiones. En cuanto a CL6 las tasas de conversión fueron mejores con donantes del tipo de cv EU1 mientras que CL5 tuvo mejores tasas de conversión con los donantes de EU11.

La menor transmisión del hipovirus entre aislados del mismo tipo de cv en el caso de EU11 hace pensar que los tratamientos de control biológico puede que no sean tan efectivos como se espera.

**6.- Factores que influyen en el crecimiento, esporulación y en la transmisión horizontal y vertical del hipovirus entre aislados del mismo tipo de compatibilidad vegetativa (V).**

Al comparar el crecimiento de los aislados hipovirulentos de Castilla y León con los hipovirus CHV1-F1 y CHV1-I se observó que existe una interacción entre el tipo de cv, el tipo de apareamiento y el hipovirus. Al igual que en estudios anteriores (Bryner y Rigling, 2012) los aislados se comportaron de forma muy diferente a 15ºC y a 25ºC. A 15ºC el crecimiento de los aislados virulentos del tipo cv EU11 siempre fue inferior al crecimiento de los aislados hipovirulentos, al contrario de lo observado con anterioridad (Peever et al., 2000; Robin et al., 2010). Los aislados que contenían el hipovirus CHV1-F1 crecieron en general más que los que contenían el subtipo italiano CHV1-I. A 25ºC los aislados hipovirulentos crecieron siempre más que los virulentos y dentro de ellos los que contenían CHV1-I en general crecieron más que los que contenían CHV1-F1. Esto confirma la influencia tanto del hongo como del hipovirus y la temperatura, como se ha visto en otros estudios (Bryner y Rigling, 2011).

En cuanto a la producción de conidios, los aislados virulentos fueron los que más conidios presentaron como era de esperar (Peever et al., 2000; Robin et al., 2010). La producción de conidios estaba influenciada por el tipo de hipovirus que portaba, siendo este el único factor significativo de los tres analizados (Hypovirus, tipo de cv y tipo de apareamiento). Los aislados hipovirulentos tanto de EU1 como de EU11 desarrollaron más esporas asexuales cuando el hipovirus que contenían era el F1. Este comportamiento no era el esperado teniendo en cuenta resultados de trabajos anteriores (Robin et al., 2010; Bryner y Rigling, 2011) donde las producciones de conidios más destacables se producen con el hipovirus italiano I. Esto parece indicar, como en el apartado anterior, que el genotipo influye.

Al analizar los aislados monospóricos obtenidos a partir de colonias que contenían el hipovirus CHV1 con los subtipos F1 e I se observó que todos los aislados hipovirulentos produjeron esporas hipovirulentas. Solamente el aislado ZA182 en combinación con CHV1-I se comportó como un aislado virulento mientras que con CHV1-F1 produjo la mayor cantidad de esporas hipovirulentas de todos los aislados testados. Esto nuevamente refuerza la teoría de que el genotipo del aislamiento influye y es muy importante seleccionar una buena combinación de hongo-virus para un control biológico exitoso.

Los ensayos de conversión nuevamente indicaron unos porcentajes de éxito de transmisión del hipovirus inferiores al 100% a diferencia de lo que aseguran trabajos anteriores (Anagnostakis y Day, 1979; Garbelotto et al., 1992; Ding et al, 2007). En este estudio, la transmisión del hipovirus entre aislados del mismo tipo de cv solo se vio afectada por el tipo de cv de los aislados.

Estos resultados indican que el potencial del control biológico en Castilla y León podría depender tanto de la selección del hipovirus como de un genotipo del hongo adecuado.

**7.- Efecto del control biológico mediante la inoculación de castaños afectados por chancro con aislados hipovirulentos (VI).**

Los aislados hipovirulentos de Castilla y León que contenían el hipovirus CHV1-F1 presentaron crecimientos similares a los aislados virulentos cuando se les inoculó en ramas en laboratorio, al contrario de lo obtenido en otros estudios (Robin et al., 2010). En el caso de los aislados hipovirulentos del tipo de cv EU11 sus crecimientos fueron menores en ramas asemejándose más al caso observado en Francia (Robin et al., 2010). Además, EU11 mostró algún efecto reduciendo el desarrollo de los chancros virulentos cuando se inocularon juntos en ramas. Sin embargo, EU1 no presentó ningún efecto en ramas.

Las inoculaciones en campo redujeron el crecimiento de la mayoría de los chancros mediante la producción de tejido cicatrizante. La efectividad de los tratamientos con hipovirulencia fue diferente en las distintas localizaciones de forma que fueron más efectivos en León (Médulas y Berlanga del Bierzo) que en Zamora (Robledo). El éxito de la transmisión de la hipovirulencia en León probablemente se deba a la presencia de aislados hipovirulentos de los dos tipos de cv más extendidos en Castilla y León (EU1 y EU11) y a la menor diversidad genética debido a que la reproducción es principalmente de tipo asexual (solo está presente MAT-1) (Anagnostakis et al., 1986; Liu et al., 2000; Cortesi et al., 2001; Papazova-Anakieva et al., 2008; Sotirowski et al., 2011). Por el contrario en Zamora no se ha encontrado hipovirulencia natural lo cual, unido a la existencia de ambos tipos de apareamiento y a la gran diversidad de tipos de cv (7 tipos de cv diferentes, Zamora et al., 2012) de esta provincia, dificulta la transmisión del hipovirus.

Los dos tipos de cv utilizados en las inoculaciones en campo dieron buenos resultados en León pero en Zamora no fueron tan efectivos puesto que solo un tratamiento de los tres empleados dentro del tipo de cv EU11 fue efectivo. La única diferencia de los inóculos de Zamora y León es que, aunque ambos pertenecían al mismo tipo de cv, el inoculo de León era MAT-1 mientras que el de Zamora era MAT-2. Estudios anteriores indican que además de los genes de incompatibilidad, la herencia genética también afecta a la transmisión del virus (Cortesi et al., 2001). Estudios recientes indican que hay genes asociados a los *loci* de incompatibilidad de *C. parasitica* que interactúan en el sistema de incompatibilidad y restringen la transmisión del hipovirus (Choi et al., 2012). Es posible que estos genes estrechamente ligados tengan influencia en la transmisión del hipovirus en el tipo de cv EU11.

En la provincia de León la presencia del hipovirus CHV1-F1 cada vez es más frecuente. Cabe pensar que en Castilla y León la enfermedad del chancro del castaño se puede controlar mediante el empleo de hipovirulencia, al menos en aquellas masas con baja diversidad de tipos de compatibilidad vegetativa, aunque se deben realizar más estudios en aquellas provincias donde el hipovirus aún no está presente.

# Conclusiones

1.- El chancro del castaño se encuentra presente en las provincias de León, Zamora, Salamanca y Ávila. La enfermedad está más extendida en León y Zamora mientras que en Salamanca y Ávila se encuentra en expansión.

2.- La diversidad de tipos de compatibilidad vegetativa de la región es relativamente alta contando con un total de once tipos de cv diferentes aunque solo dos son frecuentes, EU1(39,1%) y EU11(48,9%), mientras que los restantes son muy poco abundantes (<3%).

3.- La aparición de un solo tipo de apareamiento (Mat-1) en las provincias de León y Ávila indican que las poblaciones son clónicas y se han desarrollado a partir de tres clones (EU1, EU11 y CL5 todos Mat-1) mientras que las poblaciones de Zamora y Salamanca presentan ambos tipos de apareamiento (Mat-1 y Mat-2) lo que hace que tengan mayor reproducción sexual y más diversidad de tipos de cv.

4.- Los chancros hipovirulentos solo se han encontrado de forma natural en la provincia de León, conteniendo todos ellos el hipovirus CHV1-F1.

5.- La transmisión del hipovirus entre los aislados de *Cryphonectria parasitica* de Castilla y León fue diferente en función del tipo de cv presentando mejores resultados las conversiones de tipo de cv EU1 frente a EU11.

6.- Tanto el tipo de cv como el tipo de apareamiento y la temperatura influyen en el crecimiento de los aislados mientras que la transmisión horizontal del hipovirus solo se ve afectada por el tipo de cv. En cambio, la producción de esporas asexuales así como la transmisión vertical del hipovirus a los conidios depende del hipovirus que contenga el aislado.

7.- La presencia del hypovirus CHV1-F1 reduce la incidencia de la enfermedad del chancro en León. En Castilla y León el control biológico mediante el empleo de aislados hipovirulentos es posible. Sin embargo, es necesario dedicar más esfuerzos y hacer más estudios para conseguir reducir los efectos de la enfermedad.

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# Artículos originales

# Artículo original I

Zamora, P.; Martín, A. B.; Rigling, D.; Diez, J. J. 2012. Diversity of *Cryphonectria parasitica* in western Spain and identification of hypovirus-infected isolates. Forest Pathology 42:412-419.

***Resumen***

Diversidad de *Cryphonectria parasitica* en el oeste de España e identificación de aislados infectados por hipovirus

Los aislados del hongo causante del chancro del castaño *Cryphonectria parasitica* se obtuvieron de 44 localidades en cuatro provincias del oeste de España y se caracterizaron según los tipos de compatibilidad vegetativa (cv), tipos de apareamiento y la presencia de hypovirus 1 de *Cryphonectria* (CHV1). Entre los 1232 aislados recogidos de chancros en castaños, se determinaron 11 tipos de cv: cinco de los tipos de cv encontrados estaban incluidos dentro de la colección EU1 a EU74 (EU1, EU11, EU12, EU28 y EU66) y seis fueron tipos de cv desconocidos (CL4, CL5, CL6, CL8, CL9 y CL10). El número de tipos de cv encontrados por cada provincia varió entre dos y siete. El tipo de cv EU11 estaba presente en todas las provincias y supuso el 48.9% de todos los aislados. EU1 se encontró en tres de las provincias y supuso el 39.1% de los aislados. Los tipos de cv EU12, EU66, CL5 y CL6 estaban presentes en una o dos provincias y comprendieron entre el 2.4% y el 3% de los aislados. Los otros tipos de cv estaban representados por uno o muy pocos aislados. El tipo de apareamiento MAT-1 fue ampliamente dominante en las provincias de León y Ávila, mientras que en Salamanca y Zamora se encontraron ambos tipos de apareamiento, MAT-1 y MAT-2. Se determinaron 14 asilados hipovirulentos, nueve del tipo de cv EU1 y cinco del EU11 y todos ellos se encontraron en la provincia de León. Todos los aislados analizados contenían el hypovirus subtipo francés CHV1-F1.

Forest Pathology WILEY-BLACKWELL

For. Path. doi: 10.1111/j.1439-0329.2012.00775.x


# Diversity of *Cryphonectria parasitica* in western Spain and identification of hypovirus-infected isolates

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## Summary

Isolates of the chestnut blight fungus *Cryphonectria parasitica* were obtained from 44 localities in four provinces in Western Spain and characterized for vegetative compatibility (vc) types, mating types and the presence of Cryphonectria hypovirus 1 (CHV1). Among the 1232 isolates recovered from chestnut blight cankers, 11 vc types were identified: five known vc types included in EU1 to EU74 (EU1, EU11, EU12, EU28 and EU66) and six unknown vc types (CL4, CL5 CL6, CL8, CL9 and CL10). The number of vc types found per province varied between two and seven. The vc type EU11 was present in all provinces and accounted for 48.9% of all isolates. EU1 was detected in three provinces and accounted for 39.1% of the isolates. The vc types EU12, EU66, CL5 and CL6 were present in one or two provinces and comprised between 2.4% and 3% of the isolates. The other vc types were represented by only one or very few isolates. The mating type *MAT-1* was largely dominant in the provinces Leon and Avila, while both mating types *MAT-1* and *MAT-2* were found in Salamanca and Zamora. Fourteen hypovirus-infected *C. parasitica* isolates were found, nine were in vc type EU1 and five in EU11, and they were detected only in the province León. All isolates analysed contained the French hypovirus subtype CHV1-F1.

## 1 Introduction

European chestnut (*Castanea sativa* Mill.) is an important tree species in the region Castilla y León, growing over 67 700 ha (2º National Forest Inventory, 1986–1996) in four following provinces: León (36 000 ha), Salamanca (14 797 ha), Zamora (12 664 ha) and Ávila (3784 ha). Castilla y León holds 25% of the total chestnut fruit production in Spain which amounts to 40 000 tons per year. León produces about 8000 tons, Zamora 1000 tons and Salamanca and Ávila each 500 tons (Cesefor 2009). In Castilla y León, chestnut trees are mainly planted for fruit production but chestnut blight caused by *Cryphonectria parasitica* (Murr.) Barr. has increasingly discouraged chestnut growers because the disease reduces the profitability of the plantations and sanitation measures such as removing dried up branches and trees are time consuming.

In Europe, *C. parasitica* was first observed in Italy in 1938 on European chestnut trees (*Castanea sativa*), and from there, the pathogen has spread to most of the chestnut-growing areas in Europe (Robin and Heiniger 2001). This pathogen was very virulent and destroyed many chestnut stands before the appearance of hypovirulent strains of the fungus. This phenomenon was first detected in Italy in the 1950s and later on decline of virulence of *C. parasitica* (hypovirulence), and a recovery of the chestnut trees was observed in many European chestnut stands (Heiniger and Rigling 1994). Hypovirulence is owing to the infection of *C. parasitica* by Cryphonectria hypovirus 1 (CHV1), a unencapsidate RNA virus of the genus *Hypovirus* that results in attenuation of fungal virulence (Choi and Nuss 1992).

The hypoviruses are cytoplasmatic and can be transferred from one strain to another by hyphal anastomosis (Anagnostakis and Day 1979). Frequency and stability of hyphal anastomosis are determined by several *vic* genes that govern vegetative incompatibility (Cortesi and Milgroom 1998). This incompatibility system restricts hypovirus transmission between fungal isolates of different vegetative compatibility (vc) types, that is, individuals that have different alleles at one or more *vic* loci (Liu and Milgroom 1996; Cortesi et al. 2001). The level of vc type diversity is thought to be a critical factor for the spread of the hypovirus within *C. parasitica* populations and thus for the success of biological control using hypovirulence (Anagnostakis et al. 1986; MacDonald and Fulbright 1991; Heiniger and Rigling 1994). Therefore, diversity and distribution of vc types have been studied in important chestnut-growing areas in Europe (Cortesi et al. 1996, 1998; Homs et al. 2001; Robin and Heiniger 2001; Sotirovski et al. 2004; Perlerou and Diamandis 2006; Bragança et al. 2007; Krstin et al. 2008; Montenegro et al. 2008; Akilli et al. 2009; Robin et al. 2010). In these studies, common vc type tester strains representing 74 different vc types (EU1 to EU74) have been used (Cortesi and Milgroom 1998; Cortesi et al. 1998; Robin et al. 2000). These investigations revealed large variations in the distribution and diversity of vc types across Europe, emphasizing the need to study each chestnut-growing area individually.

The mode of reproduction of *C. parasitica* is another factor affecting the success of hypovirulence. The hypovirus is only transmitted into asexually produced pycnospores, which then can carry the virus to a new fungal individual. Sexual reproduction has a negative effect on hypovirus spread because the sexual ascospores are always hypovirus free (Carbone et al. 2004; Prospero et al. 2006). Moreover, sexual reproduction in *C. parasitica* can increase and maintain vc type diversity through the recombination of polymorphic *vic* genes (Cortesi and Milgroom 1998). As other ascomycetes, *C. parasitica* has two mating type alleles (*MAT-1* and *MAT-2*) at a single locus to control sexual compatibility (Marra and Milgroom 2001).

*Received: 30.10.2011; accepted: 13.3.2012; editor: S. Woodward*

In Spain, after the first report of chestnut blight in the province of Vizcaya (Elorrieta 1949), the disease spread throughout the northern part of the Iberian Peninsula. In Castilla y León, chestnut blight was first detected in 1978 in Bembibre (León) (De Ana Magán 1984). Currently, chestnut blight can be found in many chestnut stands in León, Zamora, Salamanca and Avila (Zamora et al. 2008a). Previous studies indicated that EU1 is the dominant vc type in the province of León followed by EU66 (Montenegro et al. 2008). Only very limited information is available for the other provinces in Castilla y León. In Zamora, the vc types EU1, EU12 and EU28 were reported (Aguín et al. 2005). In these studies, five vc types were recorded that did not belong to vc types EU1 to EU74. Since then, *C. parasitica* has spread and new outbreak sites were found in León and Zamora but also in the provinces Salamanca and Ávila, where chestnut blight was not previously detected.

The diversity and distribution of vc types, mating types and the presence of hypovirulence have to be known for an area before hypovirulence can be applied for biological control of chestnut blight. Therefore, the aim of this study was to determine the diversity of *C. parasitica* populations in Western Spain (Ávila, León, Salamanca and Zamora) through the study of vc types and mating types and to identify hypovirulent isolates through genetic analysis of the hypoviruses found in these isolates.

## 2 Materials and methods

### 2.1 Sampling

Chestnut blight was surveyed in 44 stands located in four provinces (Ávila, León, Salamanca and Zamora) in the Castilla y León region where chestnut trees are widespread (Fig. 1). In each location, between 25 and 35 chestnut blight cankers were sampled. The sampling was conducted in spring and summer between 2005 and 2009. Bark samples (5–10 $cm^2$) were removed from the edge of the cankers, and only one canker per tree was sampled. Samples were coded and kept in paper bags in portable coolers at 4°C until the isolation of the fungus in the laboratory.

### 2.2 Isolation of *Cryphonectria parasitica* from bark

For *C. parasitica* isolation, five to seven small pieces (5 × 5 mm) of bark sample were removed, dipped in 70% ethanol and flamed briefly through a Bunsen burner. The pieces were placed on potato dextrose agar (PDA; Difco, Detroit, MI, USA) plates. The plates were incubated at 24 ± 2°C in the dark for 7 days. Growing cultures were examined, and a piece of one colony per canker was transferred to a fresh PDA plate. Colonies with reduced pigmentation and sporulation were preferentially selected to increase the likelihood to recover hypovirulent isolates.

### 2.3 Identification of vc types

The vc type of the isolates was determined by assessing the barrage/merging response of pairs of isolates grown on agar medium (Anagnostakis et al. 1986). The agar medium used for vc typing was a modified PDA medium containing bromocresol-

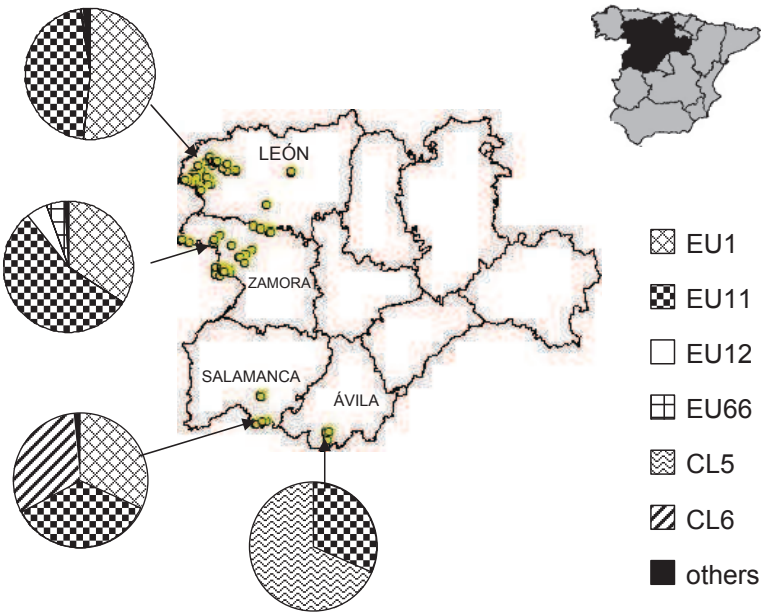


*Fig. 1.* Distribution of *Cryphonectria parasitica* vc types in the four provinces from Castilla and León where chestnut trees are present. Circles represent locations where *C. parasitica* isolates were sampled in this study. The pie charts indicate the frequencies of the most common vc types in the four provinces. Rare vc types (EU28, CL4, CL8, CL9 and CL10) were pooled into 'other' vc types.

green (Powell 1995). It consisted of 24 g PDA (Difco), 2 g yeast extract, 7 g malt extract and 0.8 g tannic acid in 1 l of distilled water. Bromocresol green (50 mg $l^{-1}$) was added to the medium to enhance the visualization of incompatible reactions (Cortesi et al. 1998). The first confrontations were made in all combinations with all the isolates in each locality and province to obtain a first collection of vc type testers in each province. After that, the testers of each province were paired with each other in all combinations to get a common collection of vc type testers for the whole region. These vc type testers from Castilla y León were then paired with the European vc type testers EU1–EU74 kindly provided by P. Cortesi and C. Robin (Cortesi and Milgroom 1998; Robin et al. 2000) to assign them to known European vc types. The diversity of vc types in each province was assessed using the Shannon and Wiener diversity index $H'$ calculated as $-\sum p_i \ln p_i$, $i = 1 \ldots S$, in which $p_i$ was the frequency of the $i$th vc type in each province and $S$ is the number of vc types in each province.

### 2.5 Mating type analysis

Mating type was determined for a subsample of 591 randomly chosen isolates. PCR amplification was conducted with the primers M1-GS1 and M1-GS2-rev for *MAT-1* and the primers M2-GS2 and InvA5n for *MAT-2* (Marra and Milgroom 1999; McGuire et al. 2001). Both primer pairs were used for each isolate. The polymerase chain reaction (PCR) was carried out in a final volume of 50 μl containing 1 μl (≈50 ng) of sample DNA, 5 μl PCR Buffer (KapaTaqBiosystem, Boston, MA, USA), 200 μM of each dNTP, 0.2 μM of each primer and 1 U *Taq*DNA polymerase (KapaTaqBiosystem). The PCR was performed in a Thermo Px2 thermocycler and consisted of an initial denaturation step at 95°C for 30 s, then 35 cycles of 30 s at 95°C, 1 min at 66°C and 4 min at 72°C, followed by a final extension at 72°C for 4 min. Amplified DNA fragments were separated by electrophoresis in 0.8% agarose gels at 67 V for 1 h.

### 2.6 Identification of hypovirulent isolates

Identification of hypovirulent *C. parasitica* isolates was based on morphological and molecular analyses. First, the isolates were assessed for morphological features typical for hypovirulent isolates such as reduced pigmentation and sporulation on PDA (Bissegger et al. 1997). Then, isolates were analysed for the presence of double-stranded (ds) RNA (i.e. the replicative form of the hypovirus) to confirm that they were infected by the hypovirus. For the dsRNA extraction, isolates were grown on PDA plates covered with cellophane for 7 days at 25°C in the dark. The cultures were removed from the cellophane sheets and transferred to 2-ml Eppendorf tubes, lyophilized and ground to a fine powder. Double-stranded RNA was isolated from 40 mg of mycelial powder by phenol/chloroform extraction followed by cellulose CF-11 chromatography (Morris and Dodds 1979; Rigling et al. 1989). After precipitation with ethanol, the dsRNA was dissolved in 20 μl of RNase-free water and stored at −20°C. The presence of dsRNA was analysed after DNase treatment in 0.8% agarose gels (Allemann et al. 1999). The Swiss CHV1-infected *C. parasitica* isolate M784 (Allemann et al. 1999) was included as a control in each set of dsRNA extraction.

### 2.7 Hypovirus sequence analysis

A specific region of the hypoviral RNA was sequenced to assign the hypoviruses found in this study to known CHV1 subtypes (Gobbin et al. 2003). Complementary DNA was synthesized from the RNA with random hexanucleotide primers using AMV reverse transcriptase (Promega, Madison, WI, USA) as described by Allemann et al. (1999). PCR amplification and sequencing were performed according to Gobbin et al. (2003). The sequences were used to construct a phylogenetic tree in the software PAUP (Swofford 2003), using the neighbour-joining method on total nucleotide differences. One reference sequence for each known CHV1 subtype was included in the phylogenetic analysis: subtype I, CHV1-Euro7; subtype F1, CHV1-EP713; subtype F2, CHV1-E57; subtype D, CHV1-E71; subtype E, CHV1-E72 (Gobbin et al. 2003).

## 3 Results

Chestnut blight was observed in all four provinces of Castilla y León where chestnut trees are very abundant. The disease was more widespread in León and Zamora than in Salamanca and Ávila where it was recently detected. A total of 1232 *C. parasitica* isolates were recovered from chestnut blight cankers and tested for vc type. Among these isolates, 11 different vc types were identified (Table 1). Five vc types were among the previously described vc types EU1 to EU74 and included EU1, EU11, EU12, EU28 and EU66. Six vc types (CL4, CL5, CL6, CL8, CL9, and CL10) were incompatible with EU1 to EU74. The main vc types were EU11 and EU1 accounting for 48.9% and 39.1% of the isolates, respectively. The other vc types were less frequent: EU12 (2.8%), EU66 (2.4%), EU28 (0.08%), CL4 (0.7%), CL5 (2.7%), CL6 (3.0%), CL8 (0.2%), CL9 (0.08%) and CL10 (0.08%).

Concerning distribution of the vc types, EU11 was isolated in all four provinces and EU1 in three of them. EU12 and CL4 were only found in León and Zamora. EU66 and EU28 appeared exclusively in Zamora but EU28 only with one isolate. CL5 was only found in Ávila where it was the most common vc type. CL6 was mostly detected in Salamanca and with few isolates also in León. The vc type CL8 comprised only three isolates in Zamora and CL9 and CL10 each only one isolate in Salamanca (Fig. 1).

A total of 591 isolates distributed among nine vc types were assayed for mating type (Tables 1 and 2). In respect to the distribution of mating types, in León and Ávila, all but one isolates in four different vc types (EU1, EU11, CL4, and CL5) were *MAT-1*. In Zamora, both mating types were found in vc type EU1 while all isolates in EU11 were *MAT-1* and all

*Table 1.* Vegetative compatibility types and mating types of *Cryphonectria parasitica* in four provinces of Castilla and León.

| Province | Number of localities | Number of isolates per vc type | | | | | | | | | | | $N^2$ | $S^3$ | $H'^4$ | n[5] | Mating types | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | EU1 | EU11 | EU12 | EU66 | EU28 | CL4 | CL5 | CL6 | CL8 | CL9 | CL10 | | | | | MAT-1 | MAT-2 |
| León | 19 | 232 (9)[1] | 205 (5)[1] | 1 | | | 7 | | 3 | | | | 448 | 5 | 0.82 | 108 | 107 | 1 |
| Ávila | 3 | | 15 | | | | | 33 | | | | | 48 | 2 | 0.66 | 45 | 45 | 0 |
| Salamanca | 4 | 35 | 39 | | | | | | 34 | | 1 | 1 | 110 | 5 | 1.17 | 104 | 61 | 43 |
| Zamora | 18 | 215 | 344 | 33 | 29 | 1 | 1 | | | 3 | | | 626 | 7 | 1.04 | 334 | 152 | 182 |
| Total | | 482 | 603 | 34 | 29 | 1 | 8 | 33 | 37 | 3 | 1 | 1 | 1232 | | | 591 | 365 | 226 |
| % of vctypes | | 39.1 | 48.9 | 2.8 | 2.4 | 0.08 | 0.7 | 2.7 | 3.0 | 0.2 | 0.08 | 0.08 | | | | | | |

[1] Number of hypovirulent isolates in brackets
[2] *N*, number of isolates
[3] *S*, number of vc types in each population
[4] *H'*, Shannon diversity index: $H' = -\sum p_i$ (Ln $p_i$) were $p_i$ in the frequency of each vc type.
[5] n, number of isolates analysed for mating types.

*Table 2.* Mating type distribution among vc types in the different provinces.

| VC Type | LEÓN | | AVILA | | SALAMANCA | | ZAMORA | |
|---|---|---|---|---|---|---|---|---|
| | *MAT*-1 | *MAT*-2 | *MAT*-1 | *MAT*-2 | *MAT*-1 | *MAT*-2 | *MAT*-1 | *MAT*-2 |
| EU1 | 86 | 0 | | | 22 | 12 | 70 | 97 |
| EU11 | 20 | 1 | 13 | 0 | 37 | 0 | 66 | 83 |
| EU12 | | | | | | | 16 | 0 |
| EU66 | | | | | | | – | – |
| EU28 | | | | | | | – | – |
| CL4 | 1 | 0 | | | | | | |
| CL5 | | | 32 | 0 | | | | |
| CL6 | | | | | 0 | 31 | | |
| CL8 | | | | | | | 0 | 2 |
| CL9 | | | | | 1 | 0 | | |
| CL10 | | | | | 1 | 0 | | |
| Total | 107 | 1 | 45 | 0 | 61 | 43 | 152 | 182 |

–, no isolates analysed for mating type.

isolates in CL6 *MAT-2*. In Salamanca, both mating types were found among isolates in vc type EU1 and EU11. All 16 isolates in the vc type EU-12 were *MAT-1.* None of the isolates yielded a PCR product with both *MAT* primer pairs.

Hypovirulent isolates were only detected in four localities in the province León (Viariz, Corullón, Melezna de los Mazos and Orellán). A total of 14 isolates, 9 belonging to vc type EU1 and 5 to EU11, had a white culture morphology on PDA typical for hypovirulent isolates (Table 1). All the hypovirulent isolates were *MAT-1*. Two isolates were lost because of contaminations before they were tested for dsRNA. The remaining 12 isolates yielded a dsRNA band of approximately 12 kbp after dsRNA extractions and agarose gel electrophoresis. Analysis of a partial sequence of the viral genome revealed a group of nine viral isolates with an identical sequence (Fig. 2). Five of them were found in the locality of Viariz (LE12, LE13, LE14, LE54 and LE56) and four in Orellán (LE171, LE172, LE175 and LE182). Three isolates differed from this group by two nucleotides, one was found in the locality of Corullón (LE46), one in Melezna de los Mazos (LE64) and one in Orellán (LE177). The phylogenetic tree showed that the 12 viral isolates grouped with CHV1-EP713, the reference hypovirus of subtype F1, and were clearly distinct from all other CHV1 subtypes (Fig. 2).

## 4 Discussion

In this study, 11 vc types were found among 1232 isolates recovered from the four provinces of Castilla y León where the chestnut culture is noteworthy. EU11 and EU1 were the most common vc types in this region, followed by EU12, EU66, CL6 and CL5. Looking at the total number of 11 vc types, Castilla y León has a relative high number of vc types but half of them (EU28, CL4, CL8, CL9 and CL10) are present in <10 isolates. Two of these rare vc types were present in Salamanca and three in Zamora.

Comparing this result with other Spanish regions surrounding Castilla y León, it can be seen that Galicia situated west of León (Fig. 3) has four different vc types (Montenegro et al. 2008). Two of them are EU1 and EU66, but the other two have no matches with the 74 European vc type testers, and it is unknown whether they are identical to some of the vc types found in Castilla y León. East of our study area, EU2 was the most widespread vc type in Cataluña (Colinas et al. 2009) and EU66 in Navarra-Pyrénée (Robin et al. 2009). The vc type EU66 is also present in Castilla y León (Zamora). This vc

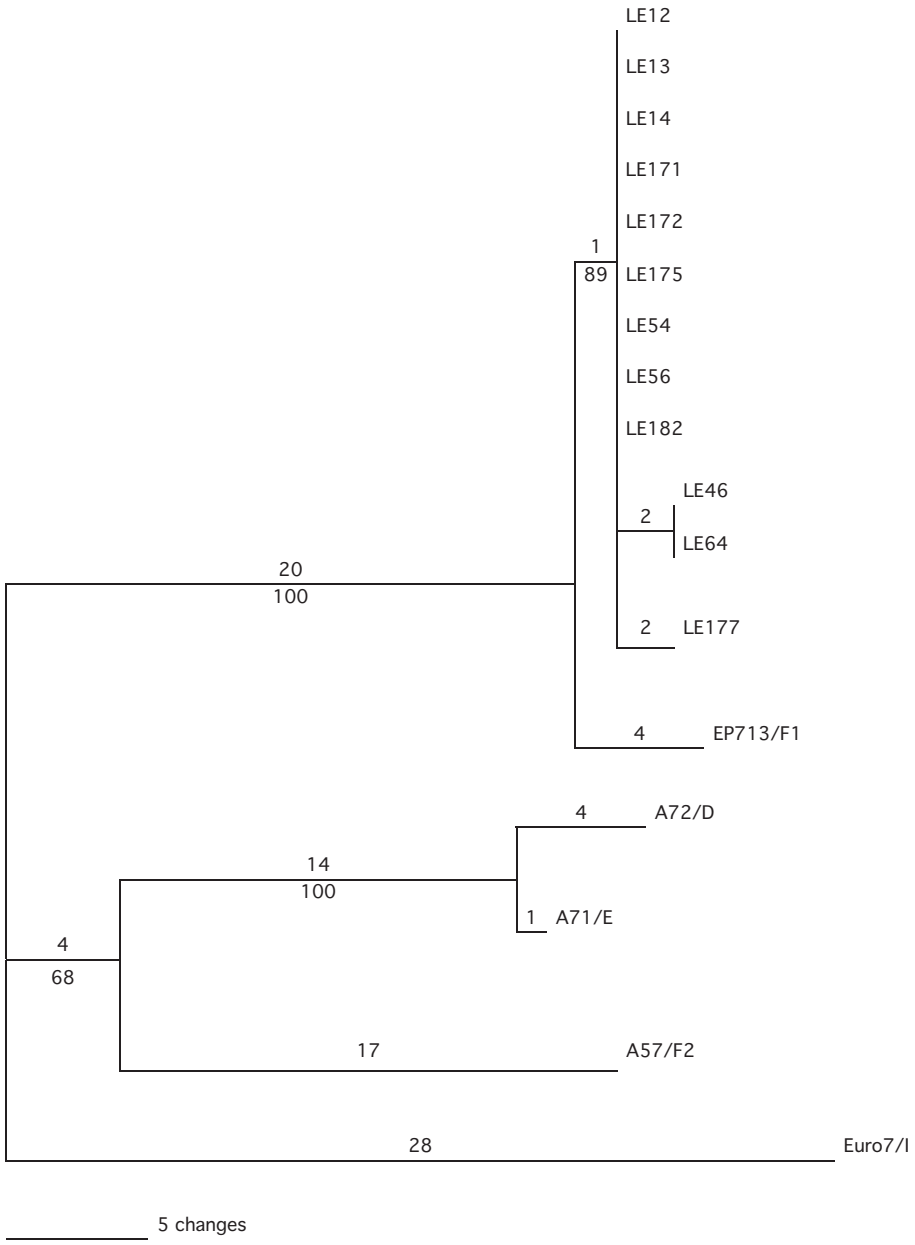


*Fig. 2.* Neighbour-joining tree derived from a partial sequence of Cryphonectria hypovirus (CHV1) isolates from Castilla y León in Spain (LE12, LE13, LE14, LE46, LE54, LE56, LE64, LE171, LE172, LE175, LE177 and LE182) and reference isolates for each known CHV1 subtype (EP713, subtype F1; A72, subtype D; A71 subtype E; A57 subtype F2; Euro7, subtype I; Gobbin et al. 2003). The number of nucleotide changes is shown above the branches and bootstrap values (1000 replications) for the main nodes below the branches.

type is not frequent in other areas of Europe except for Portugal and north-western France (Bragança et al. 2007; Robin et al. 2009). It is interesting to note that EU1 and EU2 commonly co-occur in many areas in Europe (e.g. south-eastern France, northern Italy, and Switzerland; Robin and Heiniger 2001), but only EU1 appeared in Castilla y León. This could be explained by a founder effect in which by chance only EU1 was introduced into our study area. Asturias, the region north of León, has also EU1 in common with Castilla y León but differs by having EU3 and EU13 (González-Varela et al. 2011).

In Portugal, which shares a border with Zamora and Salamanca, EU11 is the most widespread vc type followed by EU12 and EU66 (Bragança et al. 2007). EU11 is rare in Europe outside Castilla y León and Portugal being present with only a few isolates in Italy, France and Croatia (Cortesi et al. 1996; Robin et al. 2000; Krstin et al. 2008). EU12, which is the most frequent vc type in south-eastern Europe (Sotirovski et al. 2004; Perlerou and Diamandis 2006; Krstin et al. 2008; Milgroom et al. 2008), was found with several isolates in Zamora. The chestnut groves in this province are the nearest one to Portugal, and this can explain the similarities in vc types composition in these neighbouring areas. However, in contrast to Zamora and other provinces in Castilla y León, EU1 was rarely found in Portugal.

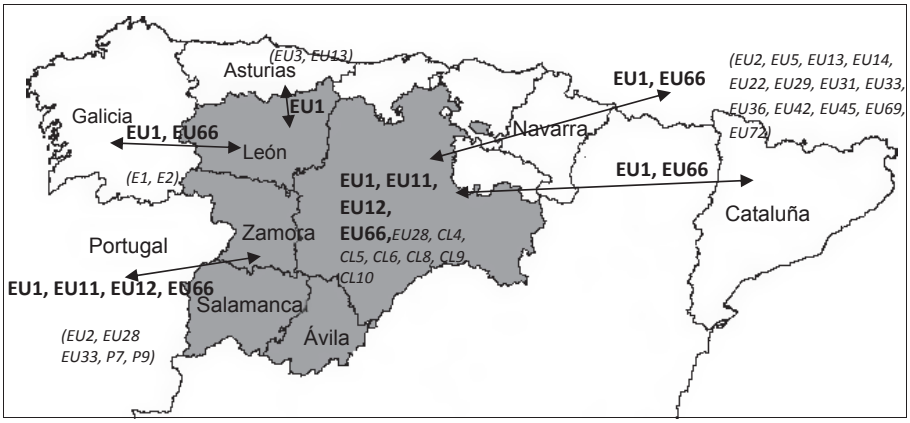


*Fig. 3.* Distribution of vc types in the surrounding regions and countries influencing the vc type distribution in Castilla y León, Galicia (Montenegro et al. 2008), Asturias (González-Varela et al. 2011), Portugal (Bragança et al. 2007), south-western France and northern Spain (Robin et al. 2009). Bold, vc types shared among regions; Italic, specific vc types in each region.

It was supposed that chestnut blight was disseminated with chestnut materials (wood and plants) transported from other Spanish regions and from France (Cobos 1989). It is likely that EU1 and EU66 have been introduced into Castilla y León along this pathway. EU11 and perhaps EU12 might have migrated from Portugal into the neighbouring Zamora and Salamanca and then further into León and Avila.

Like in a previous study with isolates from León (Montenegro et al. 2008), the most common mating type in this province was *MAT-1*. Similarly, in Ávila, we found only *MAT-1* isolates. Our study suggests that the *C. parasitica* populations in the two provinces were largely founded by three clones (EU1, *MAT-1*; EU11, *MAT-1*, and CL5, *MAT-1*). *Cryphonectria parasitica* was also found to spread as clones in expanding ranges in south-eastern Europe (Milgroom et al. 2008) and northern Switzerland (Hoegger et al. 2000). The very low incidence of *MAT-2* isolates in León and Ávila can explain the relatively low vc type diversity in these provinces despite the fact that *C. parasitica* was established during the last 30 years. The dominance of only one mating type in León and Avila indicates that *C. parasitica* mainly reproduces asexually in these areas. In populations where sexual reproduction is frequent, isolates of both mating types are expected to occur (Bragança et al. 2007; Krstin et al. 2008). This seems to be the case in Zamora and Salamanca, where *MAT-2* is almost as common as *MAT-1*. The presence of both mating types in these provinces provides the potential for an increase in vc type diversity through sexual recombination between the different vc types.

We found a very low incidence of hypovirulent *C. parasitica* isolates in the region Castilla y León. Only 14 of 1232 isolates showed the white culture morphology, typical for hypovirus-infected isolates, and all these isolates were found in only one province, León. Sequence analysis indicated that all the hypoviruses belonged to the French CHV1 subtype F1. This result supports previous investigations, in which 15 hypoviruses from León were also assigned to the CHV1 subtype F1 (Montenegro et al. 2008). None of the viral isolates was related to the Italian subtype CHV1-I, although this subtype is widespread in other areas in Europe (Allemann et al. 1999; Sotirovski et al. 2006; Krstin et al. 2008; Robin et al. 2010) and has also been found in Spain in Cataluña (Homs et al. 2001). In a *C. parasitica* isolate recovered in Navarra in 1988, an additional CHV1 subtype has been detected (Gobbin et al. 2003), but this Spanish subtype is also genetically clearly distinct from the subtype F1 found in León. Outside Spain, hypoviruses of the subtype F1 were only found in France in *C. parasitica* isolates obtained as early as 1970 (Allemann et al. 1999). Thus, it is likely that this subtype has been introduced to León from France together with its fungal hosts. Hypovirulence was also detected in northern Portugal (Bragança et al. 2007), but nothing is known about the hypovirus subtype that is present in this country. It would be interesting to analyse the hypoviruses that occur in Portugal to see whether there is any relationship with those found in our region, taking into account that the vc types are quite similar to the neighbouring country (Fig. 3). However, in the provinces closest to Portugal, Salamanca and Zamora, no hypovirulent isolates were found, suggesting that hypovirulence was probably not introduced from Portugal into Western Spain.

The low incidence of hypovirulent *C. parasitica* isolates in León could be due to the relative recent introduction of chestnut blight, which was first recorded in 1978 in this province (De Ana Magán 1984). In Europe, low level of hypovirulence has often been observed in areas where *C. parasitica* has recently established (Hoegger et al. 2000; Bragança et al. 2007). However, it remains to be seen whether the hypoviruses found in León will increase their incidence and spread to other provinces. Compared with the Italian CHV1 subtype, viruses in subtype F1 were found to greatly reduce growth and sporulation of infected *C. parasitica* strains, suggesting that they have a low ecological fitness (Chen and Nuss 1999; Robin et al. 2010). In accordance with this finding, the Italian subtype is much more widespread in Europe and typically reaches higher infection levels than the subtype F1 (Allemann et al. 1999; Krstin et al. 2008; Robin et al. 2010).

In Spain, as in other European countries, chestnut blight is present in most chestnut stands and orchards, particularly in the northern part of the country. Since the first detection of hypovirulent isolates in Spain, in Navarra and Cataluña (Allemann et al. 1999; Homs et al. 2001), more hypovirus-infected isolates have been reported in the province León (Montenegro et al. 2008; Zamora et al. 2008b). The hypovirulent isolates found were in the two most frequent vc types of the region EU1

and EU11. These two vc types together comprised 88% of the *C. parasitica* isolates recovered, suggesting a good potential for spread of hypovirulence. By providing knowledge on the distribution of vc types and mating types, our study lays the basis for a successful biocontrol programme in Castilla y León in the future. However, to reach this goal, additional studies are necessary to evaluate the performance as biological control agent of the hypoviruses found in this region.

## Acknowledgements

This study was supported by grants provided by the Regional Government of Castilla y León. We thank Dr P. Cortesi and Dr M. Milgroom for providing the EU vc type testers EU1 to EU64, and Dr C. Robin for EU65 to EU74. We also thank Esther Jung for technical assistance with hypovirus sequencing.

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Zamora, P.; Rigling, D.; Diez, J. J., 2008b: Detection of hypovirulent isolates of *Cryphonectria parasitica* in Castilla y Leon, Spain. Acta Hortic. **784**, 163–168.

# Artículo original II

Zamora, P. Martín A. B., Arrate, J., Rigling, D., Diez, J. J. 2008. Detection of the vegetative compatibility groups of *Cryphonectria parasitica* in Castilla y León region. Acta Hort. 784: 159-162.

***Resumen***

Detección de los grupos de compatibilidad vegetativa de *Cryphonectria parasitica* en la región de Castilla y León.

*Cryphonectria parasitica*, el agente causante del chancro del castaño, se observó por primera vez en Vizcaya, España en 1947 (Elorrieta, 1949). En la comunidad autónoma de Castilla y León se detectó en 1978 en Bembibre (León) (De-Ana-Magán, 1984) causando daños en sotos de castaño. Actualmente, el chancro se puede encontrar en muchas plantaciones de castaño de León y Zamora y en algunas de Salamanca y Ávila, donde *C. parasitica* se ha encontrado recientemente. Para determinar el número de grupos de compatibilidad vegetativa en Castilla y León y la distribución de estos grupos en la región, se estudiaron un total de 516 aislados. En estos aislados se identificaron un total de seis tipos de compatibilidad vegetativa (cv). Tres tipos de cv fueron compatibles con tipos de cv de la colección Europea de referencia (EU1, EU12, EU11), mientras que tres eran nuevos (CL4, CL5 y CL6). Los más ampliamente distribuidos fueron EU11 (50,6%) y EU1 (39,9%). El número y diversidad de tipos de cv en cada población fue bajo.

# Detection of the Vegetative Compatibility Groups of *Cryphonectria parasitica* in Castilla y León Region

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**Keywords:** *Cryphonectria parasitica*, chestnut blight, vc type

**Abstract**

***Cryphonectria parasitica*, the causal agent of chestnut blight, was first observed in Spain in Vizcaya in 1947 (Elorrieta, 1949). In the Autonomy of Castilla y León it was detected in 1978 in Bembibre (León) (De-Ana-Magán, 1984) causing damages on chestnut groves. At present, the chestnut blight can be found in many chestnut stands in León and Zamora and in some stands in Salamanca and Ávila where *C. parasitica* has been recently found. To determine the number of vegetative compatibility groups in Castilla y León and the distribution of these groups across the region, a total of 516 isolates of *C. parasitica* were studied. Six vegetative compatibility (vc) types were identified among the isolates. Three vc types were compatible with vc types from the European collection (EU1, EU12, EU11), whereas three were new (CL4, CL5 and CL6). The most widespread isolates were EU11 (50,6%) and EU1 (39,9%). The number and diversity of vc types in each population was low.**

## INTRODUCTION

Chestnut blight was first observed in the year 1904 in New York City on American chestnut (*Castanea dentata* Borkh.) affecting vast areas of this country thereafter (Milgroom and Cortesi, 2004). In Europe, this pathogen was first observed in Italy in 1938 on European chestnut trees (*Castanea sativa* Mill.). Since then, *Cryphonectria parasitica* has spread through most of the chestnut stand areas in Europe (Robin and Heiniger, 2001).

Although the susceptibility of *Castanea sativa* and *Castanea dentata* is similar, the chestnut blight affected the US chestnut with more intensity than the European one. This was probably due to the existence of a viral double-stranded (ds) RNA that converted the European virulent *Cryphonectria* strains to hypovirulence by the transfer of dsRNA via hyphal anastomosis (Heiniger and Rigling, 1994). This phenomenon has been used for biocontrol in various European countries with positive effects (e.g., Italy, France and Switzerland) (Heiniger and Rigling, 1994; Bissegger et al., 1997). However, the dissemination of the hypovirulence is limited in two ways. First, the hypovirus can be transmitted in variable frequencies into asexually produced conidia but not into sexual ascospores (Anagnostakis, 1988; Homs et al., 2001; Prospero et al., 2006). One other reason, is that the rate of transmission of the hypovirus is negatively correlated with the number of vegetative incompatibility (vic) genes that differ between the isolates that anastomose (Liu and Milgroom, 1996; Robin et al., 2000).

After the fist report of *C. parasitica* in Spain (Elorrieta, 1949) this pathogen has spread through the north of the Iberic Peninsula. In Castilla y León, the first detection of chestnut blight was in 1978 in Bembibre (León) (De-Ana-Magán, 1984) and currently the

chestnut blight can be found in many chestnut stands in León and Zamora and in some stands in Salamanca and Ávila where *C. parasitica* has been recently found.

The aim of the present work was to determine the number of vegetative compatibility groups in Castilla y León and their distribution across the region.

## MATERIAL AND METHODS

### Sampling and Isolation

The provinces of León, Zamora, Salamanca and Ávila were surveyed for chestnut blight and *C. parasitica* was isolated from 21 locations.

The isolation was made from bark samples taken from the cankers with a knife (one isolate per canker and tree). Small pieces from the bark samples were cut out (approx. 4-4 mm) after superficial disinfection with sodium hypochlorite (0.5%) for 30 s and with sterile water for one minute and placed on PDA (potato dextrose agar, Difco, 39 g/l). The resulting plates were incubated for 7 days at 25ºC in the dark. After that, the *C. parasitica* cultures obtained were transferred to new PDA plates using one isolate per canker for further analysis.

### Vegetative Compatibility Test

The method used to determine the vc type of the isolates was according to the barrage/merging response (Anagnostakis, 1986) and the cultivation medium was PDAg (Powell, 1995): 24 g Difco potato dextrose broth, 2 g yeast extract, 7 g malt extract, 0.8 g tannic acid, 2 mg biotin, 2 mg thiamine, 100 mg methionine and 20 g agar $L^{-1}$ ading bromocresol green (50 mg $L^{-1}$) to enhance the visualization of incompatible reactions (Cortesi et al., 1998).

The first confrontations were made in all combinations with the isolates in each province to get a first collection of vc type testers in the four provinces. After that, the resulting collections from the four provinces were tested in all combinations with each other to identify the similarities and differences in the four provinces. The resulting vc types from Castilla y León were then paired with the European vc type testers EU1-EU74. (Cortesi and Milgroom, 1998; Robin et al., 2000)

## RESULTS AND DISCUSION

A total of 516 isolates of *C. parasitica* were analysed from Castilla y León identifying 6 vc types. Three vc types were compatible with vc types from the European collection CL1=EU1, CL2=EU12, CL3=EU11) whereas three vc types were new (CL4, CL5 and CL6) (Table 1).

The most widespread isolates were EU11 (50,6% of all isolates) and EU1 (39,9%). EU11 was distributed in all four provinces, but EU1 was absent in Ávila. The other four vc types were isolated with low frequency, and with the exception of CL4 who was isolated from samples in León and Zamora, the other three vc types were found exclusively in one province (CL2=EU12 in Zamora, CL5 in Avila and CL6 in Salamanca) (Table 1).

The number and diversity of vc types in each population was low as revealed by the Shannon-Wiener index (Table 1). The total number of vc types in Castilla y León was lower than in other Spanish regions like Cataluña with 10 vc types (Homs et al., 2001) or Galicia with 8 vc types (Aguín and Romero, 2004).

## ACKNOWLEDMENTS

This research was supported by grants provided by the Regional Government of Castilla y León. We thank Dr. P. Cortesi and Dr. M. Milgroom for providing the EU vc type testers EU1 to EU64 and Dr. C. Robin for EU65 to EU74.

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## Tables

Table 1. Richness and diversity of vc types of *Cryphonectria parasitica* in Castilla y León.

| Location | VC Types | | | | | | [1]N | [2]S | [3]H' |
|---|---|---|---|---|---|---|---|---|---|
| | EU1 | EU12 | EU11 | CL4 | CL5 | CL6 | | | |
| LEÓN | | | | | | | | | |
| Corullón | 31 | | 5 | | | | 36 | 2 | 0,403 |
| Carucedo | 16 | | 40 | | | | 56 | 2 | 0,598 |
| Borrenes | 25 | | 40 | | | | 65 | 2 | 0,667 |
| Vega de Espinareda | 6 | | | 1 | | | 7 | 2 | 0,41 |
| Puente de Domingo Flórez | 23 | | 25 | | | | 48 | 2 | 0,693 |
| Priaranza | 24 | | 50 | | | | 74 | 2 | 0,63 |
| ZAMORA | | | | | | | | | |
| Hermisende | | | 23 | | | | 23 | 1 | 0 |
| Trabazos | 31 | 2 | 15 | | | | 48 | 3 | 0,758 |
| Puebla de Sanabria | | 4 | 8 | | | | 12 | 2 | 0,636 |
| Arrabalde | 27 | | | | | | 27 | 1 | 0 |
| Ayoó de Vidriales | | | | 1 | | | 1 | 1 | 0 |
| Fuente Encalada | 4 | | | | | | 4 | 1 | 0 |
| Ferreras de Abajo | | | 8 | | | | 8 | 1 | 0 |
| Ferreras de Arriba | 7 | | | | | | 7 | 1 | 0 |
| San Vitero | 1 | | 9 | | | | 10 | 2 | 0,315 |
| Rábano de Aliste | 9 | 1 | 1 | | | | 11 | 3 | 0,6 |
| SALAMANCA | | | | | | | | | |
| Linares de Riofrío | | | 23 | | | | 23 | 1 | 0 |
| Lagunilla | 1 | | | | | 12 | 13 | 2 | 0,271 |
| Montemayor del Río | 1 | | 3 | | | | 4 | 2 | 0,563 |
| ÁVILA | | | | | | | | | |
| El Arenal | | | | | 28 | | 28 | 1 | 0 |
| El Hornillo | | | 11 | | | | 11 | 1 | 0 |

[1]N = Number of isolates;

[2]S= Richness (number of vc types observed);

[3]H'=Shannon & Wiener Index of Diversity calculated with the expresion: $H' = -\Sigma pi \, Ln \, pi$. Where pi is the frequency of the $i_{th}$ vc type in each population.

# Artículo original III

Zamora, P., Rigling, D., Diez, J. J. 2008. Detection of hypovirulent isolates of *Cryphonectria parasitica* in Castilla y León, Spain. Acta Hort. 784: 163-168.

***Resumen***

Detección de aislados hipovirulentos de *Cryphonectria parasitica* en Castilla y León, España.

Los aislados de *Cryphonectria parasitica* infectados por un virus del género Hypovirus se vuelven hipovirulentos y reducen la severidad de la enfermedad del chancro del castaño. Para determinar la presencia de aislados hipovirulentos en Castilla y León se analizaron un total de 117 aislados. Los aislados se analizaron primero morfológicamente observando una pigmentación anormal y una reducción de la producción de esporas asexuales en los cultivos. Después los 117 aislados se analizaron buscando la presencia de dsRNA, el genoma del hipovirus. Se encontraron ocho aislados del tipo de compatibilidad vegetativa EU1 y cuatro del tipo EU11 que contenían dsRNA hipoviral. Todos los aislados hipovirulentos fueron de la provincia de León donde el chancro está afectando a los castaños desde 1978. Por el contrario, todos los aislados analizados de Zamora, Ávila y Salamanca, donde el chancro apareció recientemente, estaban libres de hipovirus.

# Detection of Hypovirulent Isolates of *Cryphonectria parasitica* in Castilla y León, Spain

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**Keywords:** chestnut, blight, dsRNA, hypovirus, virulence

**Abstract**

***Cryphonectria parasitica* isolates infected by viruses of the genus Hypovirus become hypovirulent and reduce the severity of the chestnut blight disease. In order to detect the presence of hypovirulent strains in Castilla y León, 117 isolates were studied. Isolates were first analyzed morphologically looking for abnormal pigmentation and reduced production of asexual sporulation in culture. After that, all 117 isolates were screened for the presence of dsRNA, the genome of the hypovirus. Eight isolates of vegetative compatibility type EU1 and four of EU11 were found to contain hypoviral dsRNA. All the hypovirulent isolates were from the León province where chestnut blight is affecting chestnut trees since 1978. In contrast, all the isolates tested from Zamora, Avila and Salamanca, where chestnut blight has appeared recently, were hypovirus-free.**

## INTRODUCTON

Infection of *Cryphonectria parasitica* by viruses of the genus Hypovirus results in attenuation of fungal virulence (Anagnostakis et al., 1998). These double-stranded (ds) RNA viruses are located in the cytoplasm of the fungus and can be transmitted from infected to uninfected strains through hyphal anastomosis (Anagnostakis and Day, 1979). The frequency and stability of hyphal anastomoses between different strains of *C. parasitica* are determined by several *vic* genes that govern vegetative incompatibility (Liu and Milgroom, 1996). For that reason, the vegetative incompatibility system in *C. parasitica* restricts the hypovirus transmission within fungal populations (Robin et al., 2000). One reason that explains the natural hypovirulence found in Europe is the low diversity of vegetative compatibility types (Bissegger et al., 1997). This natural hypovirulence is characterized by a decrease in the disease severity resulting in the recovery of many chestnut stands (Heiniger and Rigling, 1994). Hypovirulence has been applied for biological control in various European countries with positive effects (e.g., Italy, France and Switzerland) (Heiniger and Rigling, 1994).

The aim of this study was to find out if there are hypovirulent strains in the *C. parasitica* population from Castilla y León that could be used for biological control.

## MATERIAL AND METHODS

A sample of 117 isolates from areas affected by chestnut blight in Castilla y León was assayed for hypovirulence (Fig. 1; Table 1). The isolates were cultivated on potato dextrose agar medium (PDA difco, 39 g/L) for 7 days at 25ºC in the dark and 5 days on the laboratory bench with natural light. Under these conditions, CHV1-free isolates become orange pigmented with abundant asexual sporulation, whereas CHV1-infected

isolates remain white with no or few sporulation (Bissegger et al., 1997). Isolates were classified accordingly as orange, white or intermediate isolates.

All 117 isolates were subjected to dsRNA extraction to confirm the presence of hypovirus. For the dsRNA extraction, mycelium was grown on PDA plates covered with cellophane for 7 days at 25ºC in the dark. The cultures were removed from the cellophane sheets and transferred to a 2 ml Eppendorf tube, lyophilized and ground to a fine powder. Double-stranded RNA was isolated from 40 mg of mycelial powder by phenol/chloroform extraction followed by cellulose chromatography (Morris and Dodds, 1979; Rigling et al., 1989). After ethanol precipitation, the dsRNA was dissolved in 20 µl of RNase-free water and stored at -20ºC. The presence of individual dsRNA were analyzed after DNase treatment in 0,8% agarose gels. The Swiss CHV1 infected *C. parasitica* isolate M784 (Allemann et al., 1999) was included as a control in each set of dsRNA extraction.

## RESULTS AND DISCUSION

Among the 117 *C. parasitica* isolates analyzed, 100 had an orange, 11 a white and 6 an intermediate culture morphology (Table 1). Twelve of these isolates were tested positive for dsRNA. All white and one intermediate isolates proved to contain a high molecular-weight dsRNA (Fig. 2, Table 2). Eight of them were of the vegetative compatibility type EU1 and four of EU11 (Table 2). In Castilla y León region a total of six VC types have been found, three of them of an known European type (EU1, EU11 and EU12) and three of new vc type (CL4, CL5 and CL6) (Zamora, 2006 Master Thesis). The two compatibility types where the hypovirulence has been found are the most widespread in the Castilla y León region: EU1 in León, Zamora and Salamanca and EU11 in León, Zamora, Salamanca and Avila (Zamora, 2006 Master Thesis). Previously, other hypovirulent isolates have been found in Spain; one of the VC type EU2 in Cataluña (Homs et al., 2001) and one of the rare VC type EU-21 in Navarra (Allemann et al., 1999). More recently, two isolates with double stranded RNA were found in Galicia (Aguín et al., 2005).

All the hypovirulent isolates were from the province of León (Fig. 2) where chestnut blight is causing damage since 1978 (De Ana-Magán, 1984). The chestnut blight in Zamora, Avila and Salamanca provinces is more recent than in León, taking in account the survey performed by the Junta de Castilla y León since the year 2000 (Martín et al., 2001; Martín, 2003). The longer establishment of the chestnut blight in León could explain the presence of hypovirulence in this province.

The finding of hypovirulent isolates of the two most widespread VC types in Castilla y León gives a good perspective for a biological control with hypovirulence in this Spanish region.

## ACKNOWLEDMENTS

This research was supported by grants provided by the Regional Government of Castilla y León. We want to thank Hélène Blauenstein for help with the dsRNA screening.

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## Tables

Table 1. Isolates of *Cryphonectria parasitica* analyzed.

| PROVINCES | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| LEÓN | | | ZAMORA | | | ÁVILA | | | SALAMANCA | | |
| I[1] | VCG[2] | M[3] | I | VCG | M | I | VCG | M | I | VCG | M |
| LE05 | EU1 | i | ZA1 | EU12 | o | AV01 | CL5 | o | SA03 | EU11 | o |
| LE06 | EU1 | o | ZA6 | EU11 | o | AV06 | CL5 | o | SA05 | EU11 | i |
| LE11 | EU11 | o | ZA8 | EU11 | o | AV12 | CL5 | o | SA11 | EU1 | o |
| LE12 | EU1 | w | ZA10 | EU11 | o | AV18 | CL5 | o | SA16 | CL6 | o |
| LE13 | EU1 | w | ZA11 | EU1 | o | AV26 | CL5 | o | SA24 | CL6 | o |
| LE13 | EU1 | w | ZA20 | EU11 | o | AV31 | CL5 | o | SA25 | EU11 | o |
| LE14 | EU1 | i | ZA23 | EU11 | o | AV36 | EU11 | o | SA26 | EU11 | o |
| LE17 | EU1 | o | ZA24 | EU1 | i | AV40 | EU11 | o | SA27 | EU1 | o |
| LE19 | EU11 | o | ZA25 | EU1 | o | AV42 | EU11 | o | SA28 | EU11 | o |
| LE20 | EU11 | o | ZA31 | EU1 | o | AV45 | EU11 | o | SA34 | EU11 | o |
| LE21 | EU11 | o | ZA34 | EU1 | o | | | | SA40 | EU11 | o |
| LE22 | EU1 | o | ZA36 | EU1 | o | | | | | | |
| LE23 | EU11 | o | ZA38 | EU1 | o | | | | | | |
| LE24 | EU1 | o | ZA42 | EU1 | o | | | | | | |
| LE25 | EU1 | o | ZA45 | EU1 | o | | | | | | |
| LE26 | EU1 | o | ZA49 | EU1 | o | | | | | | |
| LE27 | EU1 | o | ZA53 | EU1 | o | | | | | | |
| LE28 | EU1 | o | ZA55 | EU1 | o | | | | | | |
| LE29 | EU11 | o | ZA56 | EU1 | o | | | | | | |
| LE30 | EU1 | o | ZA58 | EU11 | o | | | | | | |
| LE35 | EU1 | o | ZA63 | EU11 | o | | | | | | |
| LE36 | EU11 | o | ZA66 | EU1 | o | | | | | | |
| LE38 | EU1 | o | ZA68 | EU1 | o | | | | | | |
| LE42 | EU1 | o | ZA70 | EU1 | o | | | | | | |
| LE46 | EU1 | w | ZA73 | EU11 | o | | | | | | |
| LE48 | EU1 | o | ZA77 | EU11 | o | | | | | | |
| LE51 | EU1 | o | ZA81 | EU11 | o | | | | | | |
| LE52 | EU1 | w | ZA84 | EU11 | o | | | | | | |
| LE54 | EU1 | w | ZA87 | EU11 | o | | | | | | |
| LE56 | EU1 | w | ZA91 | EU11 | i | | | | | | |
| LE57 | EU1 | o | ZA94 | EU11 | o | | | | | | |
| LE61 | EU1 | o | ZA98 | EU11 | o | | | | | | |
| LE63 | EU1 | o | ZA103 | EU11 | o | | | | | | |
| LE64 | EU1 | w | ZA107 | EU11 | o | | | | | | |
| LE66 | EU1 | o | ZA112 | EU1 | o | | | | | | |
| LE71 | EU1 | o | ZA115 | EU1 | o | | | | | | |
| LE72 | EU1 | o | ZA118 | EU1 | o | | | | | | |
| LE73 | EU1 | o | ZA124 | EU1 | o | | | | | | |
| LE74 | EU1 | o | ZA129 | EU1 | o | | | | | | |
| LE75 | EU1 | o | ZA132 | EU1 | o | | | | | | |
| LE76 | EU1 | o | ZA135 | EU1 | o | | | | | | |
| LE81 | EU1 | o | ZA136 | EU12 | o | | | | | | |
| LE82 | EU1 | o | ZA139 | EU1 | o | | | | | | |
| LE84 | CL4 | o | ZA141 | EU1 | o | | | | | | |
| LE171 | EU11 | w | ZA147 | EU11 | o | | | | | | |
| LE172 | EU11 | w | ZA152 | EU11 | o | | | | | | |
| LE182 | EU11 | w | ZA153 | EU11 | o | | | | | | |
| LE357 | EU11 | i | ZA163 | EU12 | o | | | | | | |

[1]I: Isolate designation.
[2]VCG: Vegetative Compatibility Group.
[3]M.:Morphology (*o = orange, w = white, i= intermediate*).

Table 2. *Cryphonectria parasitica* isolates with dsRNA-infection.

| ISOLATE | LOCATION | VCG | MORPHOLOGY | dsRNA |
|---|---|---|---|---|
| LE 12 | LEÓN | EU1 | w | + |
| LE 13 | LEÓN | EU1 | w | + |
| LE 14 | LEÓN | EU1 | i | + |
| LE 46 | LEÓN | EU1 | w | + |
| LE 52 | LEÓN | EU1 | w | + |
| LE 54 | LEÓN | EU1 | w | + |
| LE 56 | LEÓN | EU1 | w | + |
| LE 64 | LEÓN | EU1 | w | + |
| LE171 | LEÓN | EU11 | w | + |
| LE 172 | LEÓN | EU11 | w | + |
| LE 182 | LEÓN | EU11 | w | + |
| LE 357 | LEÓN | EU11 | w | + |

**Figures**

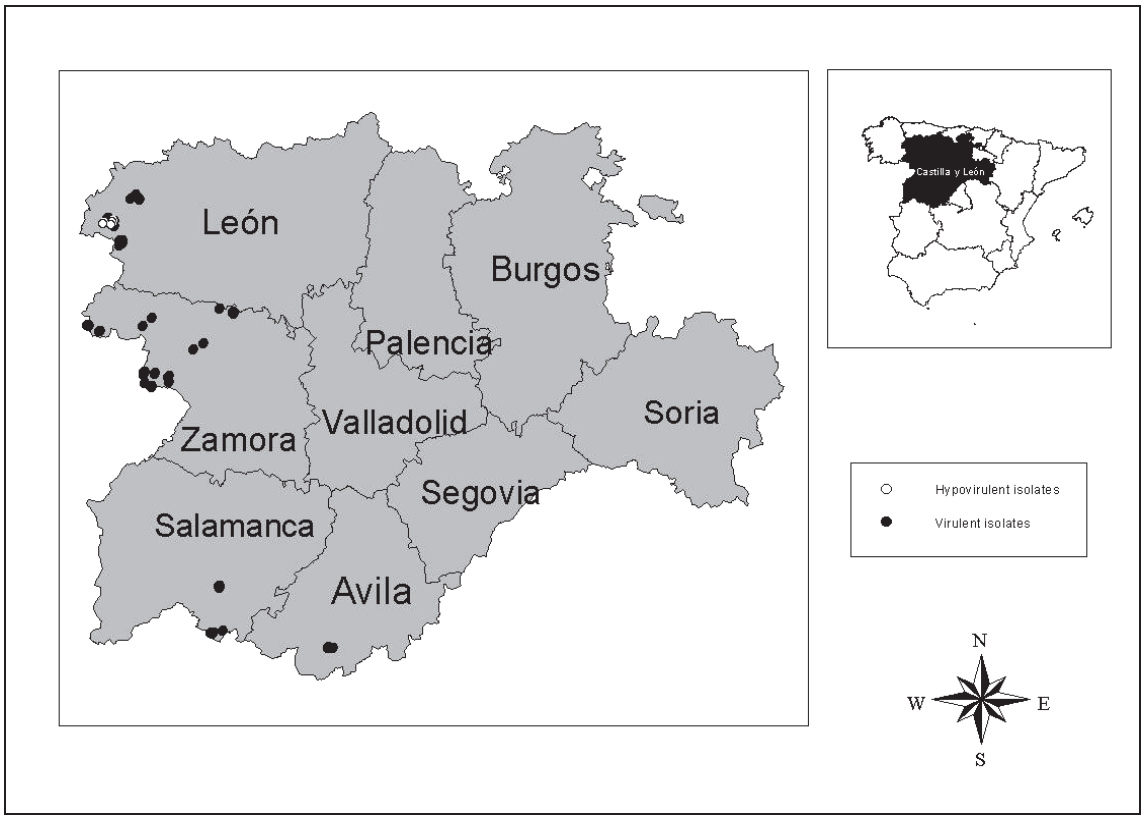


Fig. 1. Distribution of virulent (black dots) and hypovirulent (white dots) isolates in Castilla y León (Spain).

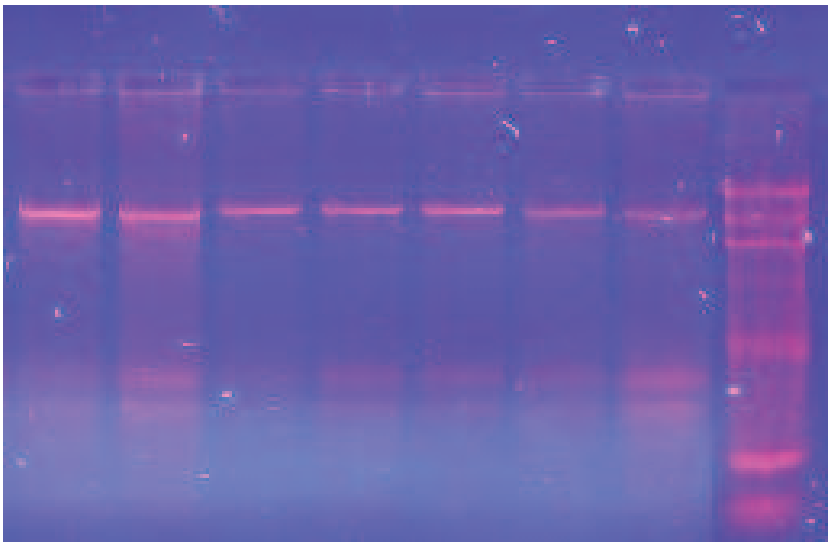

Fig. 2. Agarose gel showing seven positive results of dsRNA.

# Artículo original IV

Zamora, P.; Martín, A. B.; Dueñas, M.; San Martín, R.; Diez, J. J. 2015. *Cryphonectria parasitica* isolates of the same vegetative compatibility type display different rates of transfer of CHV1 hypovirus**.** Eur. J. Plant Pathol. DOI: 10.1007/s10658-015-0727-3

***Resumen***

Los aislados de *Cryphonectria parasitica* del mismo tipo de compatibilidad vegetativa presentan diferentes tasas de transmisión del hipovirus CHV1.

No se han encontrado aislados hipovirulentos del hongo *Cryphonectria parasitica* en la comunidad autónoma de Castilla y León (España) excepto en la provincia de León. En este estudio, hemos analizado en el laboratorio la transmisión horizontal del hipovirus CHV1 subtipo F1, aislado de masas de castaño de León. Testamos la capacidad de conversión de aislados de *Cryphonectria parasitica* de seis tipos de compatibilidad vegetativa (cv) comúnmente distribuidos en Castilla y León (EU1, EU11, EU12, EU66, CL5 y CL6). Se investigaron las tasas de conversión de aislados virulentos a hipovirulentos entre parejas de aislados pertenecientes al mismo tipo de cv (EU1, EU11 y CL6) y también se testaron las tasas de conversión cruzada entre aislados pertenecientes a tipos de cv diferentes (EU1, EU11 y CL6 como donantes y EU12, EU66, CL5 y CL6 como receptores). Los ensayos se llevaron a cabo con 1700 parejas de las cuales 700 tenían aislados donantes y receptores del mismo tipo cv y 1000 parejas de aislados con diferentes tipos cv. Los resultados mostraron que la frecuencia de conversión a aislados hipovirulentos estaba significativamente afectada por el tipo de cv, el genotipo de los aislados del mismo origen (provincia) y la interacción entre ambos factores. En la conversión entre aislados del mismo tipo de cv, las tasas de conversión fueron mejores con EU1 (rondando entre 56 y 94%) que con EU11 (variando entre 4 a 58%). En la conversión cruzada entre aislados donantes y receptores de diferentes tipos de cv, solo los receptores CL5 y CL6 fueron convertidos y la conversión de los receptores EU12 y EU66 falló en todos los casos. Con CL5 como aislado receptor, las tasas de conversión fueron similares con los donantes EU11 y CL6. Los aislados receptores de CL6 tuvieron buena conversión con aislados donantes del mismo tipo de cv (CL6) y en conversión cruzada los resultados fueron mejores con donantes EU1. Los aislados fúngicos obtenidos de masas de castaño de León presentaron las mejores tasas de conversión seguidos por Zamora, Salamanca y Ávila cuando los aislados donantes y receptores pertenecían al mimos tipo de cv. Los aislados fúngicos parecen tener una fuerte influencia en las tasas de transmisión del hipovirus, al menos entre los aislados obtenidos de las masas de castaño de Castilla y León. Los aislados de EU1 fueron más susceptibles de convertirse que los aislados de EU11. Los resultados muestran las diferencias en la transmisión del hipovirus, teniendo en cuenta los tipos de cv y el genotipo de los aislados de la misma provincia, cuando el donante y receptor son del mismo tipo de cv. Serían útiles más ensayos de transmisión para determinar por qué la transmisión del hipovirus cuando se compara con la transmisión obtenida en otros ensayos europeos, tiene diferentes tasas de conversión con los aislados de Castilla y León.

Eur J Plant Pathol
DOI 10.1007/s10658-015-0727-3

# *Cryphonectria parasitica* isolates of the same vegetative compatibility type display different rates of transfer of CHV1 hypovirus

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Accepted: 24 July 2015


**Abstract** Hypovirulent strains of the chestnut blight fungus *Cryphonectria parasitica* have not been registered in the autonomous region of Castilla y León (Spain), except in the province of León. In this laboratory-based study, we analyzed the rates of horizontal transmission of hypovirus CHV1 subtype F1, isolated from chestnut stands in León. We tested the conversion capacity of the six vegetative compatibility (vc) types of *C. parasitica* isolates most commonly distributed in Castilla y León (EU1, EU11, EU12, EU66, CL5 and CL6). We investigated conversion rates of virulent isolates into hypovirulent isolates between pairings of isolates of the same vc type (EU1, EU11 and CL6) and also tested cross conversion rates between isolates of different vc types (EU1, EU11 and CL6 as donors and EU12, EU66, CL5 and CL6 as recipients). We carried out the hypovirus transmission assay with 1700 pairings, of which 700 had donor and recipient isolates of the same vc type and 1000 pairings of isolates had different vc types. Our results show that the conversion frequency to hypovirulent isolates was significantly affected by the vc type, the genotype of isolates with the same origin (province) and the interaction between both factors. In the conversion between isolates of the same vc type, the conversion rates were better with EU1 (ranging between 56 and 94 %) than with EU11 (varying from 4 to 58 %). In the cross conversion between donor and recipient isolates of different vc types, only CL5 and CL6 recipients were converted and the conversion of recipient isolates of EU12 and EU66 failed in all cases. For CL5 as recipient isolate, the conversion rates were similar with EU11 and CL6 as the donor isolates. Recipient isolates of CL6 had good conversion with donor isolates from the same vc type (CL6) and in cross conversion the results were better with donor EU1. Fungal isolates from chestnut stands in León displayed the best conversion rates, followed by those from Zamora, Salamanca and Ávila when the donor and the recipient isolates were of the same vc type. The fungal isolates appear to have a strong influence on the transmission rate of the hypovirus, at least between strains isolated from chestnut stands in Castilla y León. EU1 isolates were more susceptible to conversion than the isolates from EU11. The results highlight the differences in hypovirus transmission, regarding vc types and the genotype of isolates from the same province, when

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Published online: 06 August 2015

donor and recipient isolates are of the same vc type. Further transmission assays would be useful to determine why hypovirus transmissions, when compared to the hypovirus transmission of other European assays, have different conversion rates with fungal isolates from Castilla y León.

**Keywords** Cryphonectria parasitica · Chestnut blight · vc type · Hypovirulence

## 1. Introduction

Horizontal transmission of pathogens between individual hosts is a key factor that affects invasion of host populations (Cortesi et al. 2001). The attenuation of the virulence of a fungal pathogen by a virus is known as hypovirulence. The transmissible hypovirulence in the chestnut blight fungus *C. parasitica* is one example of virus mediated modulation of fungal virulence. The reduction of the virulence of *C. parasitica* is caused by the infection of the fungus by a hypovirus of the family *Hypoviridae* (Van Alfen 1982; Heiniger and Rigling 1994; Hillman and Suzuki 2004; Milgroom and Cortesi 2004; Papazova-Anakieva et al. 2008; Sotirovski et al. 2011). The horizontal transmission of hypovirus has been used in the biological control of chestnut blight caused by the fungus *C. parasitica.* This virus transmission is enabled by hyphal anastomosis, whereby the cytoplasms of two fungal individuals join together and the virus is transmitted from an infected to an uninfected individual (Anagnostakis and Day 1979). Horizontal transmission of the virus is regulated by the vegetative incompatibility (vic) system of the fungus (Cortesi et al. 2001). The vegetative incompatibility system is governed by several *vic* gene loci (Cortesi and Milgroom 1998) that determine the frequency and stability of hyphal anastomosis. The probability of hypovirus transmission between incompatible individuals depends on the number and specificity of different *vic* genes in each individual (Liu and Milgroom 1996; Cortesi et al. 2001). The recent examination of vic 6 locus revealed the influence of nonallelic interactions between two tightly linked genes on virus transmission (Choi et al. 2012).

*Cryphonectria hypovirus* (CHV1) was first detected in Europe in the 1960s. Since then several subtypes have been identified (Allemann et al. 1999; Gobbin et al. 2003). The Italian subtype (subtype I) is the most widespread (Italy, Switzerland, France, Hungary, Croatia, Bosnia and Herzegovina, Macedonia, Turkey, Greece and Spain) (Allemann et al. 1999; Sotirovski et al. 2006; Krstin et al. 2008; Robin et al. 2010; Krstin et al. 2011; Akilli et al. 2013; Castaño et al. 2015). Other subtypes of CHV1 are found in Germany (subtype D), France (Subtypes F1 and F2) and Spain (Subtypes F1 and E) (Allemann et al. 1999; Gobbin et al. 2003; Zamora et al. 2012). Horizontal transmission of the virus is also influenced by the transmission ability of the CHV1 virus (Deng et al. 2009).

The first hypovirulent strains in Spain were discovered in 1988 in Navarra (Allemann et al. 1999; Gobbin et al. 2003) and in 2001 in Cataluña (Homs et al. 2001). In Castilla y León, hypovirulent strains were detected in 2005 (Montenegro et al. 2008) and in 2006 (Zamora et al. 2012). These strains appeared in a few chestnut stands in the province of León and correspond to the most extended vc types in the whole region (EU1 and EU11 infected by CHV-1 subtype F1) (Zamora et al. 2012). In the autonomous region of Castilla y León, virulent C. *parasitica* strains are also found in chestnut stands in León, Zamora, Salamanca and Ávila and some of these belong to vc types other than EU1 and EU11.

Natural biological control of the chestnut blight fungus, using the Italian subtype of hypovirus CHV1, has been successful in different European countries (Heiniger and Rigling 1994; Sotirovski et al. 2006; Krstin et al. 2008; Robin et al. 2010). Within Spain, CHV1 subtype I has been successful in controlling chestnut blight and has been widely dispersed in Cataluña (Colinas et al. 2009). Studies of the prevalence of CHV1 subtypes in France have shown that subtype F1 did not become established in the study area, unlike the Italian subtype (Robin et al. 2010). The development of hypovirulence in Castilla y León suggests that hypovirus subtype F1 is well adapted in the province of León, where both naturally extended and inoculated hypovirus strains appear to have reduced the incidence of the canker. However, the inoculations were not as effective in the orchards in Zamora (Zamora et al. 2014).

*Cryphonectria parasitica* is extended throughout most chestnut stands in Castilla y León, and there is great interest in controlling this pathogen because of the economic importance of chestnut production in the region. In natural chestnut stands in the region, hypovirulent strains of *C. parasitica* are restricted to some stands in the province of León. Therefore, the main goal of the present study was to evaluate the

conversion ability of *C. parasitica* strains in the most common vc types from Castilla y León, with the aim of optimizing transmission methods and increasing the conversion rates in the chestnut stands in the region.

## 2. Material and methods

### 2.1. Donor and recipient strains of *Cryphonectria parasitica*

To analyse the conversion capacity of the fungal strains isolated from chestnut stands in Castilla y León, we used the six most common vc types in the region (EU1, EU11, EU12, EU66, CL5 and CL6). The population structure of the fungus in the different provinces from which the isolates were collected was previously studied (Zamora et al. 2012) (Table 1). The virulent isolates used were selected from chestnut stands in the provinces of León, Zamora, Salamanca and Ávila, where inoculation trials with hypovirulent strains are of interest because of the economic significance of chestnut production. Ten virulent isolates per vc type from the different provinces were random selected (Table 2). Hypovirulent isolates were obtained in the field during analysis of the distribution of *Cryphonectria parasitica* in the province of León (Zamora et al. 2012). Four of these natural hypovirulent isolates and one isolate from Salamanca, previously transformed in the laboratory, were used as donors in the conversion test (Table 2). Both, the natural hypovirulent isolates (Zamora et al. 2012) and the one converted in the laboratory, were infected with hypovirus CHV1 subtype F1.

The method used to test transmission of the hypovirus between vc types was already described in previous studies (Anagnostakis and Day 1979; Liu and Milgroom 1996; Cortesi et al. 2001; Papazova-Anakieva et al. 2008). The transmission ability of the hypovirus was assessed by using pairs of 4×4 mm cubes of actively growing colonies of one virulent and one hypovirulent isolate of *C. parasitica*. The pairs of isolates were placed on 9-cm Petri dishes containing 20 ml of potato dextrose agar (PDA Difco) at a distance of 2–3 mm from each other and 5 mm from the edge of the plate. Five replicates of all pairings were used for assessing conversion rates to the hypovirulent phenotype. Three days after establishing the pairs and every 2 days thereafter, we evaluated the changes in colour and the morphology of the isolates. After 7 days, if the conversion had clearly taken place (morphology and colour of the recipient isolate took on that of the donor, less pigmentation and no or very weak sporulation), we removed a piece of the transformed sector of mycelia and grew it in a new Petri dish containing PDA. After 15 days, the colonies obtained from the transformed sector of mycelia, were placed under light on the laboratory bench to determine any colour changes. These isolates were subjected to dsRNA extraction to confirm the presence of hypovirus.

**Table 1** Vegetative compatibility types and mating types of *Cryphonectria parasitica* in four provinces of Castilla and León (Zamora et al. 2012)

| Province | N°of Localities | Number of isolates per vc type | | | | | | | | | | Mating Types | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | EU1 | EU11 | EU12 | EU66 | CL5 | CL6 | N[2] | S[3] | H′[4] | n[5] | MAT-1 | MAT-2 |
| León | 19 | 232 (9)[1] | 205 (5)[1] | 1 | | | 3 | 441 | 4 | 0,75 | 108 | 107 | 1 |
| Ávila | 3 | | 15 | | | 33 | | 48 | 2 | 0,66 | 45 | 45 | 0 |
| Salamanca | 4 | 35 | 39 | | | | 34 | 108 | 3 | 1,09 | 104 | 61 | 43 |
| Zamora | 18 | 215 | 344 | 33 | 29 | | | 621 | 4 | 0,99 | 334 | 152 | 182 |
| Total | | 482 | 603 | 34 | 29 | 33 | 37 | 1218 | | | 591 | 365 | 226 |
| % of vc types | | 39,1 | 48,9 | 2,8 | 2,4 | 2,7 | 3,0 | | | | | | |

[1] Number of hypovirulent isolates in brackets

[2] N: Number of isolates

[3] S: Number of vc types in each population

[4] H′: Shannon diversity index: $H' = -\sum p_i (\mathrm{Ln}\ p_i)$ where pi is the frequency of each vc type

[5] n: Number of isolates analyzed for mating types

**Table 2** Isolates (donor and recipient) of *Cryphonectria parasitica* used in the conversion analysis

| Donor and recipient isolates | | | | | | | |
|---|---|---|---|---|---|---|---|
| Province | EU1 | EU11 | CL6 | CL5 | EU12 | EU66 | dsRNa |
| Salamanca | – | – | **SA16C1*** | – | – | – | + |
| León | **LE12*** | **LE171*** | – | – | – | – | + |
| León | **LE64*** | **LE172*** | – | – | – | – | + |
| León | LE170 | LE168 | – | – | – | – | – |
| León | LE015 | LE169 | – | – | – | – | – |
| León | LE315 | LE201 | – | – | – | – | – |
| León | LE252 | LE208 | – | – | – | – | – |
| León | LE263 | LE215 | – | – | – | – | – |
| León | LE332 | LE254 | – | – | – | – | – |
| León | LE326 | LE268 | – | – | – | – | – |
| León | LE205 | LE282 | – | – | – | – | – |
| León | LE167 | LE314 | – | – | – | – | – |
| León | LE278 | LE325 | – | – | – | – | – |
| Zamora | ZA066 | ZA7 | – | – | ZA003 | ZA561 | – |
| Zamora | ZA067 | ZA12 | – | – | ZA136 | ZA564 | – |
| Zamora | ZA068 | ZA20 | – | – | ZA165 | ZA566 | – |
| Zamora | ZA069 | ZA72 | – | – | ZA185 | ZA570 | – |
| Zamora | ZA070 | ZA80 | – | – | ZA186 | ZA575 | – |
| Zamora | ZA071 | ZA140 | – | – | ZA190 | ZA578 | – |
| Zamora | ZA096 | ZA155 | – | – | ZA210 | ZA580 | – |
| Zamora | ZA198 | ZA180 | – | – | ZA213 | ZA638 | – |
| Zamora | ZA199 | ZA253 | – | – | ZA431 | ZA642 | – |
| Zamora | ZA200 | ZA320 | – | – | ZA433 | ZA643 | – |
| Ávila | – | AV033 | – | AV004 | – | – | – |
| Ávila | – | AV037 | – | AV008 | – | – | – |
| Ávila | – | AV042 | – | AV012 | – | – | – |
| Ávila | – | AV046 | – | AV016 | – | – | – |
| Ávila | – | AV050 | – | AV020 | – | – | – |
| Ávila | – | AV35 | – | AV025 | – | – | – |
| Ávila | – | AV41 | – | AV031 | – | – | – |
| Ávila | – | AV43 | – | AV051 | – | – | – |
| Ávila | – | AV65 | – | AV60 | – | – | – |
| Ávila | – | AV66 | | AV055 | – | – | – |
| Salamanca | SA011 | SA008 | SA014 | | | | – |
| Salamanca | SA027 | SA009 | SA012 | – | – | – | – |
| Salamanca | SA049 | SA010 | SA018 | – | – | – | – |
| Salamanca | SA053 | SA040 | SA022 | – | – | – | – |
| Salamanca | SA058 | SA025 | SA043 | – | – | – | – |
| Salamanca | SA45 | SA026 | SA90 | – | – | – | – |
| Salamanca | SA62 | SA006 | SA96 | – | – | – | – |
| Salamanca | SA74 | SA028 | SA100 | – | – | – | – |
| Salamanca | SA101 | SA030 | SA98 | – | – | – | – |
| Salamanca | SA104 | SA035 | SA99 | – | – | – | – |

(Bold*) Hypovirulent isolates used as donor

The conversions were made between isolates of the same vc type and also in a cross conversion with isolates of different vc types as donors and recipients. Isolates with vc types EU1 and EU11 were paired to isolates of the same vc type, in order to assess conversion to hypovirulence between isolates of the same vc type. This was done with isolates from four localizations, León, Zamora, Salamanca and Ávila. We used two hypovirulent and ten virulent isolates from each vc type and province of localization (Table 6). A total of 140 pairings were done, 60 pairing groups in EU1 and 80 in EU11. The reason for this difference between EU1 and EU11 is that in Ávila EU1 is not present. Each pairing was repeated five times and yielded a total of 700 replicates, 300 in EU1 and 400 in EU11. We used as experimental unit the five replicates of each pairing.

Isolates of vc types EU1, EU11 and CL6 were paired to isolates of vc type EU12, EU66, CL5 and CL6, in order to assess conversion to hypovirulence between isolates of different vc types. We used two hypovirulent isolates in vc types EU1 and EU11 and one in CL6 as donor isolate. The recipient virulent isolates were ten in each vc type (EU12, EU66, CL5 and CL6) (Table 6). We did a total of 200 pairings, 80 pairing groups for donor isolates from EU1, 80 for EU11 and 40 for CL6. Each pairing was repeated five times giving this a total of 1000 replicates.

### 2.2. dsRNA extraction

A dsRNA extraction procedure was carried out to confirm the presence of dsRNA in the SA16C1 isolate used as donor and the absence of dsRNA in the recipient isolates. The hypovirulent isolates LE12, LE64, LE171 and LE172 were confirmed to have dsRNA in a previous study (Zamora et al. 2012). For dsRNA extraction, mycelium was grown on potato dextrose agar (Difco) plates covered with cellophane for 7 days at 25 °C in the dark. The cultures were removed from the cellophane sheets and transferred to 2-ml Eppendorf tubes, lyophilized and ground to a fine powder. Double stranded RNA was isolated from 40 mg of mycelial powder by phenol/chloroform extraction, followed by cellulose chromatography (Morris and Dodds 1979; Rigling et al. 1989). After its precipitation in ethanol, the dsRNA was dissolved in 20 μl of RNase-free water and stored at −20 °C. The presence of dsRNA was determined after the DNase treatment, in 0.8 % agarose gels (Allemann et al. 1999). The CHV1 infected *C. parasitica* strain LE12 (Zamora et al. 2012) was included as a control in the dsRNA extraction procedure.

### 2.3. Statistical analysis

To test whether the vc type and the genotype of isolates from the same province had any effect on the hypovirulent transformation, when they were cultured with donor isolates of the same vc type, we used a mixed model. We used a mixed model because we detected a variance heterogeneity problem and the mixed model allows for a model introducing different variances for all combinations of vc type and population origin. The mixed model was represented by the following equation: $y_{ijn}=\mu+VC_i+P_j+VC_i\times P_j+\xi_{ijn}$ where $\mu$ is the overall mean, $VC_i$ the effect of the vegetative compatibility type (EU1, EU11), $P_j$ represents the province (Ávila, León, Salamanca or Zamora), $VC_i\times P_j$ the interaction between both factors, and $\xi_{ijkn}$ is the error terminus of the model. Errors were normally distributed and were independent; REML (Restricted Maximum Likelihood) variances were calculated for all combinations of vc type and population origin. The response variable, $y_{ijkn}$, is the frequency conversion (n° of pairing replicates converted/total number of pairing replicates)*100. The total number of pairing replicates was five.

To test whether the different vc types of donor and recipient isolates had any effect on the conversion to hypovirulent when co-cultured on PDA media, we used a mixed model represented by the following equation: $y_{ijn}=\mu+VCD_i+VCR_j+VCD_i\times VCR_j+\xi_{ijn}$ where $\mu$ is the overall mean, $VCD_i$ the effect of the vegetative compatibility type of the donor isolate (EU1, EU11 or CL6), $VCR_j$ represents the vegetative compatibility type of the recipient isolate (CL5 or CL6), $VCD_i\times VCR_j$ is the interaction between both factors, and $\xi_{ijn}$ is the error terminus of the model. Errors were normally distributed and were independent; REML variances were calculated for all combinations of vc types of donors and recipients. The response variable, $y_{ijn}$, is the frequency of conversion (n° of pairing replicates converted/total number of pairing replicates)*100. The total number of pairing replicates was 5.

## 3. Results

A total of 1700 pairing replicates was established: 700 with donor and recipient isolates of the same vc type and 1000 (600 of them did not convert) with different vc

types of donor and recipient isolates. The pairings that did not convert were dismissed in the statistical analysis. The principal effects show that the frequency of conversion to hypovirulence between donor and recipient isolates from the same vc type was significantly affected by the genotype of isolates from the same province and by the vc type (Table 4). Vc type EU1 (vic gene distribution 2212–22) (Table 3) displayed a higher conversion capacity than EU11 (vic gene distribution 1212–11) ($p<0.05$) (Fig. 1a). The fungal isolates from León showed the best conversion rates, followed by those from Zamora, Salamanca and Ávila when donor and recipient isolates were of the same vc type ($p<0.05$) (Fig. 1b). However, there was a significant interaction between both factors, genotype of isolates from the same province and vc type (Table 4). For transformation trials involving vc type EU1, the best results were obtained with recipient isolates from León and Zamora, with 88 and 94 % conversion frequency respectively, followed by isolates from Salamanca, with 56 % ($p<0.05$) (Fig. 2a, Table 6). By contrast, EU11 yielded good results for the virus transmission between the isolates from León (58 %), but lower transmission rates were obtained with isolates from Ávila (37 %), Salamanca (27 %) and Zamora (4 %) (Fig. 2b, Table 6).

When the conversion trials were performed with different vc types as donors and recipients, the conversion frequency of the recipient isolates into a hypovirulent strain was significantly affected by the vc type of the donor and the recipient isolate and by the interaction between both (Table 5). In the cross conversion tests with EU1 (2212–22), EU11 (1212–11) and CL6 as donors and EU12 (1112–11) and EU66 (-1-1-11) as recipients (Table 3), none of the 500 crosses yielded a recipient isolate containing the hypovirus (Table 6). Table 5 shows the results of the mixed model of conversion between donor and recipient isolates that belong to different vc types. The main factors of the cross conversion with EU1, EU11 and CL6 as donor and CL5 and CL6 as recipient show a similar frequency of hypovirus transmission with donors EU1 and CL6 and lower transmission frequency with donor EU11 (Fig. 3a) . The transmission frequency in recipient isolates of CL6 is significantly higher than the transmission frequency in recipient isolates of CL5 ($p<0.001$) (Fig. 3b). The interaction plot (Fig. 4) shows that recipient isolates of vc type CL5 had very low conversion frequency with donor isolates of CL6 (8 %) and EU11 (10 %) with no statistical differences between them ($p$-value$>0.05$). Recipient isolates of CL5 did not convert at all with donor isolates from EU1 (Fig. 4a). Recipient isolates of CL6 had a 72 % conversion frequency with the donor isolate of CL6, 44 % with donors from EU1 and 19 % with donors from EU11 and all of them were statistically different ($p$-value$<0.05$) (Fig. 4b) (Table 6).

**Table 3** VC types and vic genotype of the isolates used in the conversion assays

| VC Type | VIC Genotypes | Province* |
|---|---|---|
| EU1 | 2212-22 | León, Zamora, Salamanca |
| EU11 | 1212-11 | León, Zamora, Avila, Salamanca |
| EU12 | 1112-11 | Zamora |
| EU66 | -1-1-11 | Zamora |
| CL5 | UNKNOWN | Avila |
| CL6 | UNKNOWN | Salamanca |

Vic gene distribution: Vic genotypes with the alleles at the six known vic loci (Cortesi and Milgroom 1998). The vic genotype distribution for loci *vic 2, vic4, vic6* and *vic7* from vc type EU66 are from Peters et al., 2014. *Provinces in the region of Castilla y León where the vc types are present in chestnut populations

## Discussion

Transmission of hypoviruses in the chestnut blight fungus *Cryphonectria parasitica* isolated from Castilla y León yielded different conversion rates when isolates of the same vc type were used. In addition, cross conversion was variable and depended on the combination of vc types used in each assay. The transmission rates were highly variable among the fungal isolates from the four provinces in Castilla y León (Zamora, Salamanca, León and Ávila) and also within the different vc types studied.

Conversion between strains of the same vc types failed to reach 100 %. Although conversion from a virulent to a hypovirulent strain is known to occur rapidly by hyphal anastomosis when both isolates are of the same vc type (Liu and Milgroom 1996; Cortesi et al. 2001; Ding et al. 2007; Papazova-Anakieva et al. 2008; Bryner and Rigling 2012). This was not always the case here. In the conversion with donor and recipient isolates of the same vc type, the rate of conversion obtained with isolates of vc type EU1 from León and Zamora was similar to other studies and lower in the

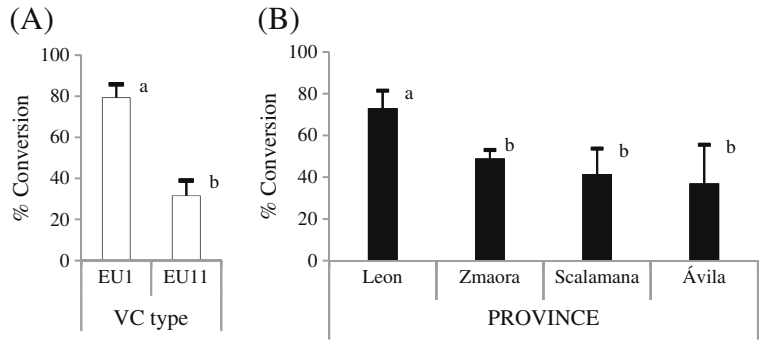


**Fig. 1** Principal effects. Conversion frequency between hypovirulent and virulent strains of *C. parasitica* belonging to the same vc type: EU1 or EU11 (**a**) and belonging to one province from Castilla y León (**b**). The province represents the different genotype of the isolates from the same origin. The following formula was used for the calculation: % conversion=(n° of pairing replicates converted/total number of pairing replicates)*100. Error bars represent 95 % confidence intervals

case of fungal isolates from Salamanca (56 %). When the conversion was between isolates of EU11, the rate of conversion decreased greatly and was very variable, ranging from 4 to 58 %. This vc type is not frequent in Europe, except in Portugal (Bragança et al. 2007; Zamora et al. 2012), and it would be interesting to carry out transmission assays with vc type EU11 of Portuguese origin. Nothing is known about other cases with limited horizontal transmission of virus, when donor and recipient strains are of the same vc type. Recent studies support that the transmission capability assessed in vitro in the laboratory underestimates the in situ ability of CHV1 to migrate within a fungal population composed of different vegetative compatible types (Ding et al. 2007; Brusini and Robin 2013). The difference in transmission frequency between the laboratory and the field experiments indicates that perhaps in living trees there is enough time before the host cells dies for the effective transfer of rival dsRNA (Ding et al. 2007). The transmission ability of CHV1 in vc type EU11 might be higher in situ than in vitro, even if the transmission is between isolates from the same vc type and there is no cell death caused by the incompatible recognition of isolates from different vc types. This is supported by the results of the first inoculation trial with chestnut trees in the region of Castilla y León, which achieved a good conversion rate with isolates from EU11 in stands in the province of León (Zamora et al. 2014).

**Table 4** Linear model of the frequency of conversion to hypovirulence with donor and recipient strains of the same vc type. Error df=133

| Source | df | F | Pr>F |
|---|---|---|---|
| vc type | 1 | 92.61 | <.0001 |
| Province | 3 | 10.63 | <.0001 |
| vc type*Province | 2 | 27.95 | <.0001 |

The efficiency of conversion with vc type EU11 was clearly different in the fungal isolates from the four populations of the study areas. The conversion of vc type EU11 was relatively high with strains from León (58 %) and it was much lower with strains from Ávila (37 %), Salamanca (27 %) and Zamora (4 %). Comparing the fungal strains from the four populations, the distribution of mating types indicates that *C. parasitica* populations from Zamora and Salamanca, which include both mating types, are more diverse than those from León and Ávila, in which only Mat-1 is found (Zamora et al. 2012). In addition to the *vic* genes, the host genetic background has been shown to affect virus transmission (Cortesi et al. 2001). In clonal populations, the transmission of the virus can take place more readily between individuals due to the lack of genetic diversity (Milgroom et al. 2008; González-Varela et al. 2011). This may explain the better conversion rate with isolates from León, which included a small number of different vc types and only one idiomorph (Mat-1) (Zamora et al. 2012). The lower transmission success in vc type EU11 from Zamora may be influenced by a higher degree of genetic variability in this vc type than in isolates from stands in the other provinces in Castilla y León. In a previous study (Zamora et al. 2012), the distribution of idiomorphs Mat-1 and Mat-2 in vc type EU11 showed that both, Mat-1 and Mat-2, were only present in isolates from Zamora, in contrast to the isolates from the other

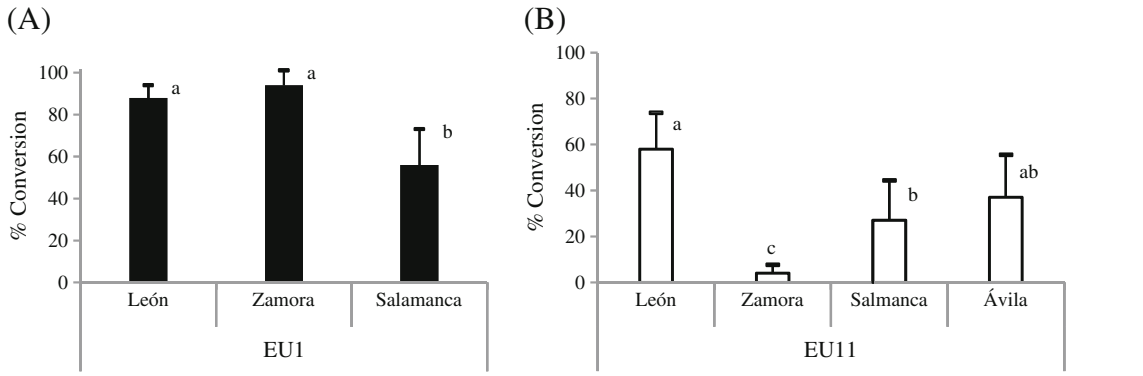


**Fig. 2** Interaction plot. Conversion frequency of the isolates from vc type EU1 (**a**) and EU11 (**b**) belonging to the different provinces. The following formula was used for the calculation: % conversion=(n° of pairing replicates converted/total number of pairing replicates)*100. Error bars represent 95 % confidence intervals

provinces, in which only Mat-1 was found (Zamora et al. 2012). This supports the suggested higher genetic variability in Zamora due to recombination events via sexual reproduction.

Other studies have demonstrated the influence of the hypovirus type on the ability to transform virulent strains (Bryner and Rigling 2011; Sotirovski et al. 2011). We used hypovirulent isolates from chestnut stands in León, which contained hypovirus CHV1 subtype F1 (Zamora et al. 2012). This hypovirus is supposed to convert very efficiently, reducing pigmentation and sporulation, and its use is recommended for therapeutic treatment of individual cankers, as for example in high-value orchards or plantations (Robin et al. 2010). The limited spread, distribution and adaptation of the French hypovirus in the field is due to the severe effect of this hypovirus on *C. parasitica* (Robin et al. 2010); however, it might be possible that the conversion is not as efficient as assumed and that it could actually depend on the vc type of the fungal strain. In contrast, the most widespread hypovirus in Europe is CHV1 subtype I from Italy, which is better adapted to the environment (Allemann et al. 1999; Dawe and Nuss 2001; Bryner et al. 2012). It would be interesting to determine, whether the Italian CHV1 subtype I has the same effect in isolates/strains of vc type EU11 of the fungus. The probability of transmission was recently analysed with three subtypes of hypovirus CHV1 (D, F1 and I) (Bryner et al. 2012). Different transmission rates were observed between donor and recipient strains that were heteroallelic at one *vic* loci; with isolates of the same vc type, transmission was almost 100 %, and the genetic background had some influence. Hypovirus subtype F1 yielded the highest rate of transmission. This contrasts with the low transmission rates obtained in vitro with hypovirus subtype F1 and vc type EU11 from Castilla y León, even when vc type EU11 was used as both donor and recipient strain of the fungus. The influence of the genetic background of the fungal isolates from Castilla y León may also explain the uneven transmission of the hypovirus. Donor and recipient isolates have influence on the hypovirus transmission. Although this influence has been studied, the main factor considered were the differences in the allelic distribution of the *vic* loci (Cortesi et al. 2001). Recently, the characterization of the vic 6 locus revealed the involvement of nonallelic interactions between two tightly linked genes on virus transmission, as well as their influence on the barrage formation and heterokaryon formation (Choi et al. 2012).

Conversion using different vc types as donor and recipient isolates was not successful with EU12 and EU66 as recipient strains. Although EU12 and EU11 are only heteroallelic at one *vic* locus, all of the conversion trials were negative. Heteroallelism for these two vc

**Table 5** Linear model of the frequency of conversion to hypovirulence with donor and recipient strains of different vc types. Error df=75

| Source | df | F | Pr>F |
|---|---|---|---|
| vc type_Donor | 2 | 8.36 | 0.0005 |
| vc type_Recipient | 1 | 33.07 | <.0001 |
| vc type_D*VCG_R | 1 | 18.77 | <.0001 |

**Table 6** Conversion rates of the fungal isolates from chestnut stands in the four provinces of Castilla y León, where *C. parasitica* occurs

| vc type Donor | N° Donor isolates[a] | vc type Recipient | N° Recipient isolate[b] | Province[c] | N° pairing replicates[d] | N° Replicates. convert/total[e] | %Conv.[f] |
|---|---|---|---|---|---|---|---|
| EU1 | 2 | EU1 | 10 | León | 5 | 88/100 | 88 |
| EU1 | 2 | EU1 | 10 | Zamora | 5 | 94/100 | 94 |
| EU1 | 2 | EU1 | 10 | Salamanca | 5 | 56/100 | 56 |
| EU11 | 2 | EU11 | 10 | León | 5 | 58/100 | 58 |
| EU11 | 2 | EU11 | 10 | Zamora | 5 | 4/100 | 4 |
| EU11 | 2 | EU11 | 10 | Salamanca | 5 | 27/100 | 27 |
| EU11 | 2 | EU11 | 10 | Ávila | 5 | 37/100 | 37 |
| EU1 | 2 | CL6 | 10 | Salamanca | 5 | 44/100 | 44 |
| EU11 | 2 | CL6 | 10 | Salamanca | 5 | 19/100 | 19 |
| CL6 | 1 | CL6 | 10 | Salamanca | 5 | 36/50 | 72 |
| EU1 | 2 | CL5 | 10 | Ávila | 5 | 0/100 | 0 |
| EU11 | 2 | CL5 | 10 | Ávila | 5 | 10/100 | 10 |
| CL6 | 1 | CL5 | 10 | Ávila | 5 | 4/50 | 8 |
| EU1 | 2 | EU12 | 10 | Zamora | 5 | 0/100 | 0 |
| EU11 | 2 | EU12 | 10 | Zamora | 5 | 0/100 | 0 |
| CL6 | 1 | EU12 | 10 | Zamora | 5 | 0/50 | 0 |
| EU1 | 2 | EU66 | 10 | Zamora | 5 | 0/100 | 0 |
| EU11 | 2 | EU66 | 10 | Zamora | 5 | 0/100 | 0 |
| CL6 | 1 | EU66 | 10 | Zamora | 5 | 0/50 | 0 |

a: Number of hypovirulent isolates used with each vc type as donors b: Number of recipient isolates c: province of origin of the recipient isolates d: Number of replicates with each combination of one hypovirulent donor isolate and one virulent recipient isolate e: number of successes divided by the number of trials f: frequency of conversion

types occurs at *vic*2, which is known to greatly inhibit the virus transmission (Cortesi et al. 2001). Also, the transmission trials with EU66 as the recipient strain failed in all replicates with isolate donors of both vc types EU1 and EU11; however, when CL5 and CL6 were used as recipients, some isolates became hypovirulent. These two vc types do not correspond to the collection of 72 European vc types, and nothing is known about the allele relation between these and vc types EU1 and EU11. The CL6 isolates appeared to

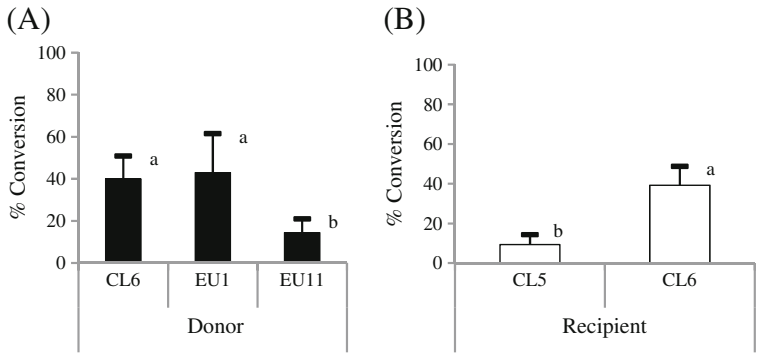


**Fig. 3** Principal effects. Conversion frequency between hypovirulent and virulent strains of *C. parasitica* belonging to different vc types: conversion frequency with donor strains of vc types CL6, EU1 and EU11 (**a**) and conversion frequency in recipient strains of vc types CL5 and CL6 (**b**). The following formula was used for the calculation: % conversion=(n° of pairing replicates converted/total number of pairing replicates)*100. Error bars represent 95 % confidence intervals

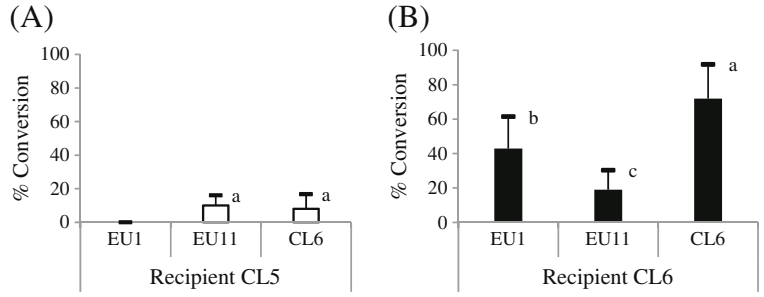


**Fig. 4** Interaction plot. Conversion frequency between hypovirulent and virulent strains of *C. parasitica* belonging to different vc types: conversion frequency in recipient strains of CL5 (**a**) and CL6 (**b**) with donor isolates of vc types EU1, EU11 and CL6. The following formula was used: % conversion=(n° of pairing replicates converted/total number of pairing replicates)*100. Error bars represent 95 % confidence intervals

yield better results in the transmission of the hypovirus with vc type EU1 as donor (44 %); with EU11 as donor, some of the isolates also became hypovirulent (19 %). In contrast, with CL5 as recipient, the conversion rate was lower and only effective with EU11 (10 %) or CL6 (8 %) as donor strains, but not with EU1 (0 %). In some cases, the transmission of hypovirus has been registered to occur between incompatible strains in the field, although the virus transmission between these strains did not occur in the laboratory (Deng et al. 2009).

The transfer rate of hypovirus CHV 1 was different regarding vc types and the genotype of isolates from the same province, when donor and recipient strains were of the same vc type. The low rates of cross conversion are of concern. Further transmission assays would be useful to determine why hypovirus transmissions, when compared to the hypovirus transmission of other European assays, have different conversion rates with fungal isolates from Castilla y León.

**Acknowledgments** This research was supported by grants provided by the Regional Government of Castilla y León and the project "*Control biológico del chancro del castaño en Castilla y León*" VA053A08.

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# Artículo original V

Zamora, P.; González Casas A.; Dueñas, M.; San Martín, R.; Diez, J. J.. Factors influencing growth, sporulation and virus transfer of *Cryphonectria parasitica* isolates from Castilla y León (Spain). Enviado a Eur. J. Plant Pathol.


***Resumen***

Factores que influyen en el crecimiento, esporulación y transferencia de virus de aislados de *Cryphonectria parasitica* de Castilla y León (España)

El control biológico con *Cryphonectria hypovirus* CHV1 ha reducido el impacto del chancro del castaño en Europa. Este hipovirus causa el debilitamiento de los aislados del hongo de forma que el hongo produce chancros no letales permitiendo a los castaños superar la enfermedad. El virus se puede transmitir horizontalmente a través de la anastomosis hifal o verticalmente a los conidios. Se testó la capacidad de crecimiento, esporulación y transmisión horizontal y vertical del virus a diferentes temperaturas. Para determinar el comportamiento diferente de los aislados fúngicos, utilizamos aislados de los dos tipos de compatibilidad vegetativa (cv) más ampliamente distribuidos en Castilla y León y ambos tipos de apareamiento, Mat-1 y Mat-2. Y para determinar la influencia del virus, los aislados se parasitaron con dos subtipos de hipovirus CHV1-F1 y CHV1-I. El crecimiento y la transmisión horizontal se determinaron a dos temperaturas diferentes 15ºC y 25ºC. El análisis del crecimiento mostró una interacción entre el tipo de cv, el tipo de apareamiento y el hipovirus a 15ºC y 25ºC. Pero la transmisión horizontal solo estaba influenciada por el tipo de cv y la producción de esporas solo por el hipovirus. La transmisión vertical del virus además estaba influenciada por el aislados fúngico y el virus. Todos los aislados hipovirulentos de EU1 produjeron gran cantidad de conidios hipovirulentos que desarrollaron colonias con micelio blanco y aéreo y poca producción de conidios, típico de aislados infectados por hipovirus. Pero los aislados de EU11 se comportaron como aislados hipovirulentos intermedios cuando pertenecían al tipo de apareamiento Mat-1. Los aislados EU11 de Mat-2 produjeron conidios hipovirulentos solo con el subtipo de hipovirus CHV1-F1. Los resultados muestran diferente comportamiento de los aislados fúngicos con ambos subtipos de hipovirus siendo más eficiente en general los aislados parasitados por F1 que aquellos con el subtipo I al contrario que en otros estudios. Esto confirma la teoría de que el hospedante fúngico y el virus tienen una gran influencia en la transmisión y diseminación de la hipovirulencia. El genotipo del hongo tiene una gran influencia en la tasa de crecimiento y en la transmisión horizontal del virus y el virus controla la producción de esporas y la transmisión del virus a la progenie. Por ello, la selección del aislado fúngico y del hipovirus es muy importante para el éxito del control biológico.

## Factors influencing growth, sporulation and virus transfer of *Cryphonectria parasitica* isolates from Castilla y León (Spain).

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**Summary**

The biological control with *Cryphonectria hypovirus* CHV1 has reduced the impact of chestnut blight in Europe. This hypovirus causes weakness in the fungal strain in a way, that the fungus causes non-lethal cankers and allows the chestnut trees to overcome the disease. The virus can be transmitted horizontally by hyphal anastomosis or vertically to the conidia. The capability of growth, sporulation and horizontal and vertical transmission of the virus at different temperatures was tested. To determine the different behavior of the fungal strain, we used isolates from the most widespread vegetative compatibility types (vc types) in Castilla y León, both mating types, Mat-1 and Mat-2. To determine the influence of the virus the isolates were parasitized with two hypovirus subtypes CHV1-F1 and CHV1-I. Growth and horizontal transmission were assessed at two different temperatures 15ºC and 25ºC. The growth analysis showed an interaction between VC type, Mating type and hypovirus at 15ºC and 25ºC. But the horizontal transmission was only influenced by the vc type and the spore production only by the hypovirus. The vertical transmission of the virus was also influenced by the fungal isolate and the virus. All the hypovirulent isolates from EU1 produced high amounts of hypovirulent conidia that produced colonies with the white and aerial mycelium and few conidia production, typical of hypovirus infected isolates. But the isolates of EU11and mating type Mat-1, behaved like intermedia hypovirulent isolates. The fungal isolates EU11 Mat-2 produced hypovirulent conidia only with the hypovirus subtype CHV1-F1. The results show a different behavior of the fungal isolates with both hypovirus subtypes in contrast to other studies, being more efficient, in general, the isolates that were parasitized with F1 than the ones parasitized with subtype I. This confirms the theory that fungal host and virus greatly influence the hypovirulence transmission and dissemination. The fungal genotype has a big influence in growth rate and horizontal transmission of the virus and the virus controls the spore production and the vertical transmission of the virus to the progeny. Therefore the selection of the fungal isolate and the hypovirus is very important for the success of biological control.

## 1.- Introduction

The biological control of the chestnut blight canker, caused by the fungus *Cryphonectria parasitica*, has reduced the impact of the disease in Europe (Robin and Heiniger, 2001). This biological control is due to the *Cryphonectria hypovirus 1* (CHV), an unencapsidate RNA virus, that causes weakness in the fungal strains and produces non-lethal cankers allowing chestnut trees to overcome the disease (Nuss1992; Newhouse, 1990; Turchetti 1982). The hypovirus transmission can occur horizontally via hyphal anastomosis to a new host or vertically to the progeny (Pearson et al., 2009; Brusini and Robin, 2013). The hyphal anastomosis is controlled by several vic genes that govern vegetative incompatibility (Cortesi and Milgroom, 1998). This incompatibility system restricts the transmission of the virus between fungal isolates reducing the impact of the biological control if the vc type diversity is high (Liu and Milgroom, 1996; Cortesi et al., 2001). Although the horizontal virus transmission occurs between isolates of the same vc types, occasionally the transmission can occur between different vc types when the genotypes of the interacting host individuals are closely related (Liu and Milgroom, 1996; Cortesi et al., 2001). In Europe the vc type distribution is relatively low, allowing the success of biological control. This is in contrast to North America where the vc type diversity is very high (Milgroom and Cortesi, 2004).

Vertical transmission allows the spread of the virus within its host across longer distances (Brusini and Robin, 2013). But the hypoviruses reduce asexual spore production (Choi and Nuss, 1992; Heiniger and Rigling, 1994; Peever et al., 2000; Robin et al., 2000, Brusini and Robin, 2013), reducing the spread of virus to new areas. In addition the proportion of infected conidia is dependent on the hypovirus type (Lin et al., 2007; Castaño et al., 2015). In Europe the most frequent hypovirus is CHV1 with five subtypes: CHV1-I, CHV1-F1, CHV1-F2, CHV1-D and CHV1-E) (Allenmann et al., 1999). From these, the most frequently found is CHV1-I, found in Italy, Switzerland, Croatia, Bosnia-Herzegovina, Hungary, Greece, Corsica, Slovenia, Macedonia, France, Turkey and Spain (Alemman et al., 1999; Homs et al., 2001, Sotirovski et al., 2006; Robin et al., 2010; Krstin et al., 2011; Akilli et al., 2013; Castaño et al., 2015).

Furthermore the sexual reproduction is a handicap in the biological control because sexual ascospores are always free from hypovirus and this sexual reproduction involves a recombination of polymorphic vic genes increasing the VC type diversity (Cortesi and Milgroom, 1998; Carbone et al., 2004; Prospero et al., 2006). The sexual reproduction is controlled by two mating type alleles (Mat-1 and Mat-2) (Marra and Milgroom, 2001) and the presence of both mating types implies the increase of diversity via sexual reproduction. The distribution in Europe of these mating types shows a more abundant presence of Mat-1 in some countries including Spain (Sotirovski et al., 2004, Perlerou and Diamandis, 2006; Montenegro et al., 2008; Aguín et al., 2008; González-Varela et al., 2011; Zamora et al., 2012; Risteski et al., 2013). This uneven distribution contributes to a better dissemination of the hypovirus, controlling the chestnut blight disease progression. The environment is another factor of influence in the *Cryphonectria* pathosystem. One important variable the temperature (Bryner and Rigling, 2011). This factor could help to select the apropiate season to do the field inoculations to ensure the best outcomes in the biological control.

In Castilla y León the distribution of *Cryphonectria parasitica* is concentrated in four provinces, Zamora being the most diverse of them (Zamora et al., 2012). In Zamora we found a large distribution of VC types EU1 and EU11 and the presence of isolates from both mating types, Mat-1 and Mat-2. On the other hand, the hypovirus distribution in Castilla y León is still

very low, and only the province of León has hypovirulent isolates (Zamora et al., 2012). Although in Spain the most frequent hypovirus is CHV1-I, in the region of Castilla y León the only hypovirus subtype found to date was CHV1-F1. In this study we compared the influence of two hypovirus subtypes, CHV1-F1 and CHV1-I, in different isolates of the most extended VC types, EU1 and EU11, and both mating types, Mat-1 and Mat-2, in order to evaluate the influence of the hypovirus and the fungal isolates in the growth, spores production and hypovirus horizontal and vertical transmission at different temperatures. This allows for the selection of the best candidate for use in the biological control of *Cryphonectria parasitica*.

## 2.- Materials and methods

### 2.1.- Isolates of *Cryphonectria parasitica*

The isolates used to analyse differences in growth, sporulation and horizontal transmission of hypovirus were from the two most common vc types in Castilla y León (EU1, EU11)**.** The virulent isolates selected belong to diverse populations from Zamora, where the chestnut canker has more variability throughout the region. Four virulent isolates per vc type were selected, two of each mating type (Table 1) and transformed to hypovirulent. These fungal isolates were used as virulent and hypovirulent, combined with CHV1 subtypes F1 which is naturally distributed in the region (Zamora et al., 2012) and CHV1 subtype I, not found in Castilla y León (Table1). To assess the presence or absence of hypovirus, an extraction of dsRNA was conducted for all the isolates and analysed by the CF-11 cellulose purification procedure (Morris and Dodds, 1979; Rigling et al., 1989).

### 2.2.- Growth on culture media at 15 and 25°C

To check differences in growth of the virulent and hypovirulent isolates, in vc type EU1 we used isolates ZA205 from mating type Mat-1 and ZA444 from mating type Mat-2. For vc type EU11 we used ZA478 from mating type Mat-1 and ZA 182 from Mat-2. The fungus-virus combination was done with these fungal isolates and the hypovirus was transmitted from infected fungal isolates LE12 containing CHV1-F1 (Zamora et al., 2012) and LL0998 with CHV1-I (Castaño et al., 2015) via hyphal anastomosis (Table 1) (Anagnostakis and Day 1979; Liu and Milgroom 1996; Rigling et al. 1989; Peever et al. 2000; Cortesi et al. 2001; Papazova-Anakieva et al. 2008; Robin et al., 2010; Zamora et al. 2012). Five replicates for each virulent and hypovirulent isolate were grown under 25ºC and the same number under 15ºC, in dark conditions. The colonies were obtained placing a 5 mm piece of mycelia from five days old cultures in the middle of the plate. All plates were stored in the incubation chamber by 25ºC and 15ºC after seiling them with parafilm. The plates were kept in the growing chambers for 10 days and every 24 hours two cardinal diameters from each colony were measured with a millimetric ruler in two orthogonal axes. The ellipse area was used to determine the differences in growth. To compare the growth rate of the different isolates we used the day before most growing isolates reached the border of the petri dish.

### 2.3.- In-vitro horizontal transmission at 15 and 25°C

The capability of the fungal isolates to become hypovirulent through the horizontal transmission of the hypovirus via hyphal anastomosis was measured with isolates of the same

vc type (EU1 and EU11). For this experiment we used the same donor isolates used in the growth assay, for vc type EU1: ZA205 and ZA444, and for vc type EU11: ZA478 and ZA182. All the donor isolates had two copies, one with hypovirus subtype F1 and one with subtype I (Table 1). The recipient isolates were four virulent isolates, two from each mating type in both vc types: ZA66 - ZA198 (Mat-1, EU1), ZA540 - ZA630 (Mat -2, EU1), ZA591 - ZA697 (Mat - 1, EU11) and ZA620 -ZA698 (Mat -2, EU11) (Table 1). The method used to assess the transmission of the hypovirus between vc types was previously described (Anagnostakis and Day 1979). Two, five millimeter mycelial plugs, one hypovirulent and one virulent were placed in a 90mm diameter petri dish with potato dextrose agar (PDA, Difco Laboratories). These were separated from each other by 4 millimeters and were 5 mm from the edge of the plate. For each donor- recipient combination five replicates were done and all the plates were stored in the growing chamber at 25ºC or 15ºC for 15 days in darkness after seiling them with parafilm. Every 24 hours after the first three days the plates were checked for virus transmission. This transmission was detected from the morphological change of the virulent colony assembling the hypovirulent one. When a change was observed a piece of the changed sector was cultured in a new petri dish with potato dextrose agar media (PDA, Difco Laboratories) for further analysis to confirm hypovirulence.

**2.4.- Sporulation and phenotype of monosporic cultures**

For each fungal isolate from the experiments a group of five replicates were cultured on potato dextrose agar (PDA, Difco Laboratories) to assess the sporulation capability. These plates were illuminated by fluorescent tubes (Phillips MasterTL-D30W/830) with a photoperiod of 14h and a light intensity of 2500 lx. The temperature was of 25ºC ±2 ºC. The sporulation was assessed 15 days after the incubation by adding 5 ml of water with a drop of Tween 80 (Technical-Prolabo) to each colony to liberate the conidia. Each spore suspension was diluted four times ($10^{-1}$, $10^{-2}$, $10^{-3}$ and $10^{-4}$) and then quantified counting the spores of 25µl suspension with a hemocytometer (Thoma, 0.1mm, 0.0025mm$^2$) under a light microscope whith 40x magnification. The measured concentration for the analysis was used as conidia/ml.

Ten monosporic colonies were done from each replicate used in the spore production assay (50 monosporic colonies for each fungal isolate). We put a drop of spores from the spore suspension in potato dextrose agar (PDA, Difco Laboratories) and left them growing in a chamber at 25ºC in dark conditions for 48 hours. After two days we selected ten single colonies under binocular magnifying glass with ground lighting with 63x magnification and put every single colony in a new petri dish with potato dextrose agar. Those new monosporic colonies were grown for 7 days in a chamber at 25ºC in dark conditions and 10 days in the laboratory bench with natural illumination. After this time the colonies were analyzed for morphologic differences following those described previously by Garbelotto et al.1992: pigmentation, conidia production and mycelium morphology. All colonies were classified in three groups: Hypovirulent (H) those with white pigmentation, aerial mycelium, with less or no apparent conidia production, Intermedia (I) with pale orange pigmentation with few sporulation and not so fluffy but not compact mycelium, Virulent (V) those with the typical virulent morphology, with orange pigmentation, a lot of fruiting bodies and compact mycelium. At least three of the resulting colonies of each isolate and each group (H, I and V) were analysed for dsRNA presence or absence.

### 2.5.- Statistic analysis

To assess the differences in growth, conversion and sporulation we used mixed models. For the growth analysis at 15 and 25 ºC the mixed model was represented by the following equation:

$Growth_{ijkn} = \mu + H_i + VC_j + Mat_k + H_i\,x\,VC_j + H_i\,x\,Mat_j + VC_j\,x\,Mat_k + H_i\,x\,VC_j\,x\,Mat_k + \xi_{ijkn}$ where $\mu$ is the overall mean, $H_i$ in the hypovirus effect (F1, I, V), $VC_j$ the effect of the vegetative compatibility type (EU1, EU11), $Mat_k$ the effect of the mating type (Mat-1, Mat-2) and all the interactions. The $\xi_{ijkn}$ is the random error of the model. Error was normally distributed, independent and with different variance for each hypovirus, VC type and mating type combination. The temperature was not included in the model because these two temperatures have a clear influence in growth.

The horizontal transmission of the hypovirus was assessed with a mixed model from the factors $H_i$ that represent the hypovirus effect (F1, I, V), $VC_j$ the effect of the vegetative compatibility type (EU1, EU11), $Mat\_D_k$ and $Mat\_R_l$ the effect of the mating type from the donor and the recipient isolates (Mat-1, Mat-2) and $T_m$ the temperature effect and all the interactions until level 3. The random error of the model had a normal distribution, independent and with different variance for each hypovirus, VC type and mating type combination.

The following equation for the mixed model was used to compare the conidia production:

$Ln\,(1+ conidia_{ijkn}) = \mu + H_i + VC_j + Mat_k + H_i\,x\,VC_j + H_i\,x\,Mat_k + VC_j\,x\,Mat_k + H_i\,x\,VC_j\,x\,Mat_k + \xi_{ijkn}$ where $\mu$ is the overall mean, $H_i$ is the hypovirus effect (F1, I, V), $VC_j$ the effect of the vegetative compatibility type (EU1, EU11), $Mat_k$ the effect of the mating type (Mat-1, Mat-2) and all the interactions. Error was normally distributed, independent and with different variance for each hypovirus, VC type and mating type combination. The logarithm was used because the number of conidia per ml was very high and the expression (1+conidia) was used to consider the zero results.

The monosporic characterization, the association between different isolates and the morphological classification were done with a Simple Correspondence Analysis (SCA). The Cluster Analysis was done using the Ward method and the square Euclidean distance. Homogeneous groups that were obtained were characterized from the different variables: VC type , Mating type , hypovirus and isolates.

### 3.- Results

All main factors (VC type, Mating type and Hypovirus) and interactions affected significantly the growth of the colonies at 15ºC ($p \leq 0,05$) (Figure 1a, Table 2) and at 25ºC (Figure 1b, Table 3). When the temperature was 15ºC the isolates of mating type Mat-1 grew more when they were from vc type EU1 in contrast to those corresponding to EU11. The same occurred with Mat-2, VC type EU1 had more growth than EU11 but the difference was somewhat less. The hypovirulent isolates with CHV1-F1 grew more than those of CHV1-I in exception of isolates from EU11 and Mat-2 where CHV1-F1 and CHV1-I had the same growth. When the temperature was 25ºC the isolates of Mat-1 EU1 grew more than those of Mat-1 EU11 and the same occurred with Mat-2 in exception of isolates of CHV1-F1 (Figure 1b). In contrast, at 25ºC isolates with CHV1- I grew more than those with CHV1-F1. The virulent

isolates grew clearly less than the hypovirulent isolates at 25ºC but at 15ºC virulent isolates from VC type EU1 grew more in case of Mat-1 or the same those of Mat-2.

The main factors in the conversion assay were VC type, Mating type of the donor and recipient isolates and the hypovirus but only the VC type affected significantly the conversion of the isolates. With both temperatures, 15ºC and 25ºC, the isolates of VC type EU1 showed better conversion rates than those belonging to EU11 ($p \leq 0,05$) (Figure 2 a, b, table 4 and table 5).

The main factors in the sporulation assay were VC type, Mating type and hypovirus. In this assay only the hypovirus affected the spore production of the different isolates (Figure 3, Table 6). The virulent isolates were the most productive of all, followed by isolates with CHV1-F1 and CHV1- I.

The Simple Correspondence Analysis and Cluster analysis grouped the monosporic isolates in three different groups. The first group where the virulent isolates and ZA182I, the second group of intermediate virulence was composed by ZA478 with both hypovirus subtypes F1 and I. The third group of hypovirulent isolates included the remaining hypovirulent isolates (Figures 4 and 5).

**4.-Discussion**

In this study we found an interaction of the three main factors (VC type and mating type of the fungal host and the hypovirus parasitizing the host) in the fungal isolates growth. As in previous studies (Bryner and Rigling, 2012) the fungal isolates have different growth at 15 and 25ºC. The interactions at 15ºC show different behavior of isolates from VC type EU1 and EU11. When the isolates were from VC type EU1 the virulent isolate grew more or equal to the hypovirulent isolates (CHV1 subtype F1 and I). This is in-line with other studies (Peever et al., 2000; Robin et al., 2010) but the growth of virulent isolates from VC type EU11 was always less than the growth obtained with the hypovirulent ones at 15ºC. At this temperature hypovirulent isolates with CHV1-F1 grew, in most cases, more than those with CHV1-I. However at 25ºC the hypovirulent isolates grew more than the virulent ones and those with hypovirus subtype I better than with F1 except for EU11 Mat-2. This reinforces the idea of the influence of the fungal isolate, hypovirus and temperature on the growth behavior (Bryner and Rigling, 2011). Several studies have analysed the growth of *Cryphonectria parasitica* isolates comparing between the different hypovirus and in some cases with the virulent isolates (Peever et al., 2000; Robin et al., 2010; Bryner and Rigling 2011) but nothing is known about the difference between isolates corresponding to Mat-1 or Mat-2. Isolates of Mat-1 grew more than those of Mat-2, both virulent and hypovirulent at 15ºC. The population in different Spanish regions is dominated by mating type Mat-1 like in some provinces from Castilla y León (Montenegro et al., 2008; Zamora et al., 2012), Asturias (González-Varela et al., 2011) and Galicia (Aguín et al., 2008).This uneven distribution where Mat-1 is more abundant also occurs in other European countries like Macedonia, Greece or Bulgaria (Sotirovski et al., 2004, Perlerou and Diamandis, 2006; Risteski et al., 2013). This better growth of idiomorph Mat-1 could explain the greater expansion of Mat-1 in contrast to Mat-2 in different orchards. But at 25ºC the isolates behaved different with more growth of Mat-2 especially the hypovirulent isolates. The different behavior of the hypovirulent isolates in both mating types indicates that the potential of biological control may vary between the different virus-fungus combination and temperatures, as said before (Bryner and Rigling, 2011; Bauman, 2015). In case of a biological

control it would be interesting to select the best fungal isolate, taking into consideration the temperature, the distribution of both VC and Mating types of the cankers to be inoculated and the hypovirus to parasite the fungal isolate.

The capacity of transmission of the hypovirus between two fungal isolates was influenced only by the vc type of the fungus, regardless of the hypovirus or the mating type of the fungal host. The conversion was less effective in isolates of VC type EU11 than EU1, as demonstrated before (Zamora et al., 2015). The horizontal transmission of *Cryphonectria hypovirus* CHV1 is affected by virus strains when the transmission is between isolates heteroalellic at one or two vic loci (Deng et al., 2009) Other authors found no differences in the transmission comparing different hypovirus (Liu and Milgroom, 1996; Cortesi et al., 2001). In this study, the transmission of the hypovirus between isolates of the same vc type was not influenced by the hypovirus as expected but by the vc type of the fungal isolate. This opens the question whether the virus transmission is more influenced by the fungal isolate than by the virus strain. The transmission between two isolates of the same vc type is always supposed to be successful but this was not the case of EU11 isolates. This, added to lack of differences between the hypovirus tested, indicates that the fungal isolate has more influence in the transmission than was previously assumed The other factor analysed was the temperature, but although it had a big influence in the growth rate of the different isolates, the horizontal transmission of both hypovirus subtypes was similar at 15ºC and 25ºC. This similarity in conversion for both hypovirus and temperatures, allows the field inoculation to be performed during different seasons of the year and with both hypovirus subtypes. It would only be necessary to be aware of the characteristics of the fungal isolates like growth, spore production and amount of hypovirulent conidia produced by the fungus.

The first inoculation trial done in the field in the province of Zamora showed good results with one of three isolates of vc type EU11 and mating type Mat-2. In contrast, the inoculations were better in León with isolates EU11 and Mat-1 (Zamora et al., 2014). This different behavior made us suspect that there could be differences of virus transmission ability between both mating types. But the results show that, at least in the laboratory, both mating types have the same capability of virus transmission between isolates of the same VC type.

In contrast, the spore production was influenced by the hypovirus producing more spores with CHV1- F1 than with the Italian subtype I. This was unexpected considering previous studies where the production of spores was more efficient with CHV1-I (Bryner and Rigling, 2011; Robin et al., 2010). The variances in the behavior of the hypovirus subtypes in the different studies indicate again an important influence of the fungal isolate. In Castilla y León it seems that the hypovirus subtype CHV1-F1 is not able to decrease intensively the conidia production of *C. parasitica* and is therefore a better candidate for the biological control than the Italian subtype CHV1-I, which is more extended in other European countries (Allemann et al., 1999; Sotirovski et al., 2006; Robin et al., 2010).

The monosporic colonies showed big differences with isolate ZA182 when the fungus was parasitized with the CHV1-F1 or CHV1-I. The first one, ZA182F1, produced the largest amount of hypovirulent spores and, in contrast when the fungus was in combination with CHV1-I, the spore production was mainly virulent. This different behavior among isolates of EU11 and mating type Mat-2 makes the hypovirus selection very important because it will

determine the success of the dissemination of the hypovirus in the orchards after the inoculations. In contrast, isolate ZA478 was the only one that showed an intermediate appearance in the morphology of the colonies with both hypovirus CHV1-F1 and I. This intermediate hypovirulent morphology makes it difficult to distinguish virulent from hypovirulent isolates and the amount of hypovirulent isolates is perhaps underestimated. However, EU1 isolates had similar hypovirulent colonies production regardless the hypovirus or the mating type of the fungal isolate.

Grouping all the data obtained, the fungal isolates behaved differently with both hypovirus subtypes with a general better results (more growth, sporulation and hypovirulent conidia production) of subtype F1 in contrast to other studies (Robin et al., 2010, Bryner and Rigling., 2011, Deng et al., 2009; Bauman et al., 2015). This reinforces the theory that fungal host and virus have a big influence in the hypovirulence transmission and dissemination (Bryner and Rigling, 2011; Bauman, 2015). The fungal isolate has a big influence in growth rate and horizontal transmission of the virus and the virus controls the spore production and the vertical transmission of the virus to the progeny. Therefore the selection of the vc type and mating type of the fungal isolate and the hypovirus is very important for the success of the biological control.

**Acknowledgements**

This research was supported by grants provided by the Regional Government of Castilla y León.

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Table 1.- Fungal isolates used in the different assays. The virulent isolates in the second part of the table were used as receptor isolates in the conversion assay.

| | | | | Origin of the Hypovirus | |
|---|---|---|---|---|---|
| **Isolate** | **Mat** | **VC** | **HV** | **Donor isolate** | **Donor VC** |
| ZA205 | MAT-1 | EU1 | - | - | - |
| ZA205F1 | MAT-1 | EU1 | F1 | LE12 | EU1 |
| ZA205I | MAT-1 | EU1 | I | LL0998 | EU1 |
| ZA444 | MAT-2 | EU1 | - | - | - |
| ZA444 F1 | MAT-2 | EU1 | F1 | LE12 | EU1 |
| ZA444I | MAT-2 | EU1 | I | LL0998 | EU1 |
| ZA478 | MAT-1 | EU11 | - | - | - |
| ZA478 F1 | MAT-1 | EU11 | F1 | LE172 | EU11 |
| ZA478I | MAT-1 | EU11 | I | LL0998 | EU1 |
| ZA182 | MAT-2 | EU11 | - | - | - |
| ZA182 F1 | MAT-2 | EU11 | F1 | LE182 | EU11 |
| ZA182I | MAT-2 | EU11 | I | LL0998 | EU1 |
| ZA066 | MAT-1 | EU1 | - | - | - |
| ZA198 | MAT-1 | EU1 | - | - | - |
| ZA540 | MAT-2 | EU1 | - | - | - |
| ZA620 | MAT-2 | EU1 | - | - | - |
| ZA591 | MAT-1 | EU11 | - | - | - |
| ZA697 | MAT-1 | EU11 | - | - | - |
| ZA620 | MAT-2 | EU11 | - | - | - |
| ZA698 | MAT-2 | EU11 | - | - | - |

Isolate: name of the fungal isolate from the Castilla y León collection of isolates (Zamora et al., 2012), MAT: Mating type of the isolate, VC: VC type of the isolate, HV: Hypovirus present in the isolate, Donor isolate: isolate used previously to transfer the hypovirus to create the fungus-virus combination for the assays, Donor VC: VC type of the donor isolates used for the transfer of hypovirus.

Table 2.- Linear model of growth at 15ºC

| **Source** | **Num df** | **Den df** | **F** | **Pr > F** |
|---|---|---|---|---|
| VC | 1 | 48 | 135,71 | <.0001 |
| MAT | 1 | 48 | 75,2 | <.0001 |
| VC*MAT | 1 | 48 | 16,99 | 0,0001 |
| HIPOVIRUS | 2 | 48 | 6,9 | 0,0023 |
| HIPOVIRUS*VC | 2 | 48 | 23,2 | <.0001 |
| HIPOVIRUS*MAT | 2 | 48 | 3,35 | 0,0435 |
| HIPOVIRUS*VC*MAT | 2 | 48 | 4,88 | 0,0118 |

Table3.- Linear model of growth at 25ºC

| **Source** | **Num df** | **Den df** | **F** | **Pr > F** |
|---|---|---|---|---|
| VC | 1 | 48 | 47,28 | <.0001 |
| MAT | 1 | 48 | 32,85 | <.0001 |
| VC*MAT | 1 | 48 | 29,98 | <.0001 |
| HIPOVIRUS | 2 | 48 | 99,04 | <.0001 |
| HIPOVIRUS*VC | 2 | 48 | 27,26 | <.0001 |
| HIPOVIRUS*MAT | 2 | 48 | 19,83 | <.0001 |
| HIPOVIRUS*VC*MAT | 2 | 48 | 39,14 | <.0001 |

Table 4.- Linear model of conversion rate at 15ºC

| Source | Num df | Den df | F | Pr>F |
|---|---|---|---|---|
| Hypovirus | 1 | 17 | 0,03 | 0,8565 |
| **VC** | **1** | **17** | **13,94** | **0,0017** |
| Hypovirus*VC | 1 | 17 | 0,09 | 0,7633 |
| Mat_D | 1 | 17 | 0,3 | 0,5889 |
| Hypovirus*Mat_D | 1 | 17 | 1,35 | 0,2609 |
| VCG*Mat_D | 1 | 17 | 0,45 | 0,5098 |
| Hypoivurs*VC*Mat_D | 1 | 17 | 0 | 0,9519 |
| Mat_R | 1 | 17 | 0 | 0,9519 |
| Hypovirus*Mat_R | 1 | 17 | 0,03 | 0,8565 |
| VC*Mat_R | 1 | 17 | 0,63 | 0,4371 |
| Hypoivurs*VC*Mat_R | 1 | 17 | 0 | 0,9519 |
| Mat_D*Mat_R | 1 | 17 | 0 | 0,9875 |
| Hypovirus*Mat_D*Mat_R | 1 | 17 | 0,04 | 0,8506 |
| VC*Mat_D*Mat_R | 1 | 17 | 0,04 | 0,843 |

Table 5.- Linear model of conversion rate at 25ºC

| Source | Num df | Den df | F | Pr>F |
|---|---|---|---|---|
| Hypovirus | 1 | 17 | 1,49 | 0,2385 |
| **VC** | **1** | **17** | **16,58** | **0,0008** |
| Hypovirus*VC | 1 | 17 | 1,18 | 0,2927 |
| Mat_D | 1 | 17 | 0 | 1 |
| Hypovirus*Mat_D | 1 | 17 | 1,18 | 0,2927 |
| VC*Mat_D | 1 | 17 | 0,9 | 0,3554 |
| Hypoivurs*VC*Mat_D | 1 | 17 | 0,17 | 0,689 |
| Mat_R | 1 | 17 | 3,61 | 0,0745 |
| Hypovirus*Mat_R | 1 | 17 | 0,29 | 0,5942 |
| VC*Mat_R | 1 | 17 | 0,17 | 0,689 |
| Hypoivurs*VC*Mat_R | 1 | 17 | 0,17 | 0,689 |
| Mat_D*Mat_R | 1 | 17 | 0,17 | 0,687 |
| Hypovirus*Mat_D*Mat_R | 1 | 17 | 1,15 | 0,2985 |
| VC*Mat_D*Mat_R | 1 | 17 | 0,32 | 0,5804 |

Table 6.- Linear model of spore production

| Source | Num df | Den df | F | Pr > F |
|---|---|---|---|---|
| **Hipovirus** | **2** | **48** | **9,06** | **0,0005** |
| VG | 1 | 48 | 0,03 | 0,8597 |
| Hipovirus*VG | 2 | 48 | 1,87 | 0,1645 |
| MAT | 1 | 48 | 0,02 | 0,8823 |
| Hipovirus*MAT | 2 | 48 | 1,57 | 0,218 |
| VG*MAT | 1 | 48 | 0,26 | 0,6109 |
| Hipovirus*VG*MAT | 2 | 48 | 0,3 | 0,7427 |

Figure 1.- Growth at 15ºC (a) and 25ºC (b) of isolates of VC types EU1 and EU11, Mating type Mat-1 and Mat-2 containing CHV1 F1 and I and without hypovirus(V) . The growth was calculated with the area of the colonies in $mm^2$. Error bars represent 95% confidence intervals.

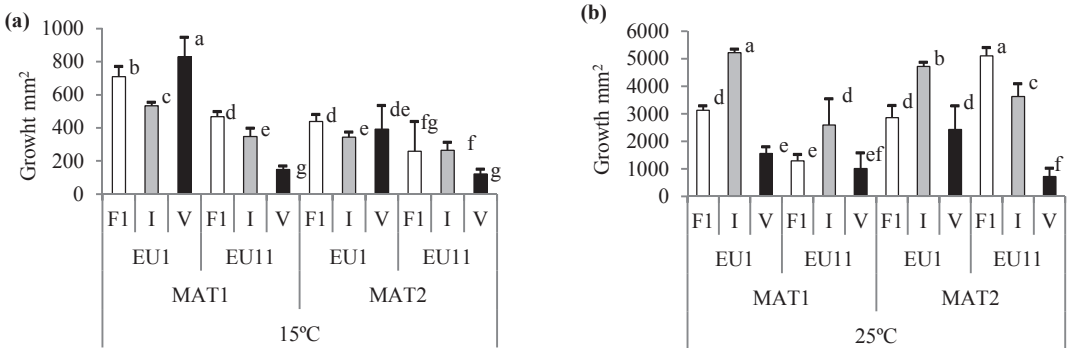



Figure 2.- Conversion frequency at 15ºC (a) and 25ºC (b) between hypovirulent and virulent strains of *C. parasitica* belonging to vc types EU1 and EU11. The following formula was used: % conversion = (nº of pairing replicates converted/total number of pairing replicates)*100. Error bars represent 95% confidence intervals.

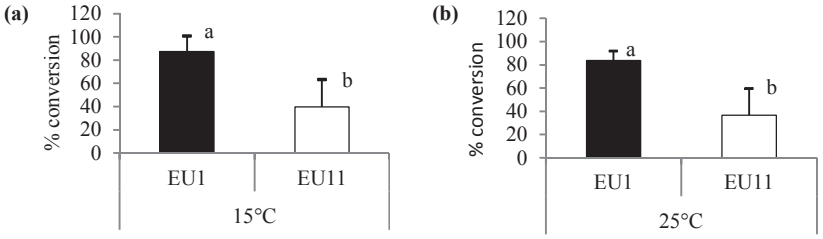



Figure 3.- Sporulation differences between virulent (V) and hypovirulent isolates containing CHV1 subtypes F1 and I. Error bars represent 95% confidence intervals.

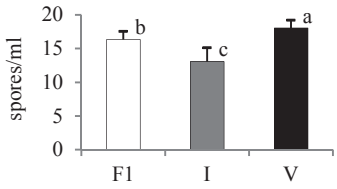



Figure 4.- Simple Correspondence Analysis plot. Factor 1 versus Factor 2.

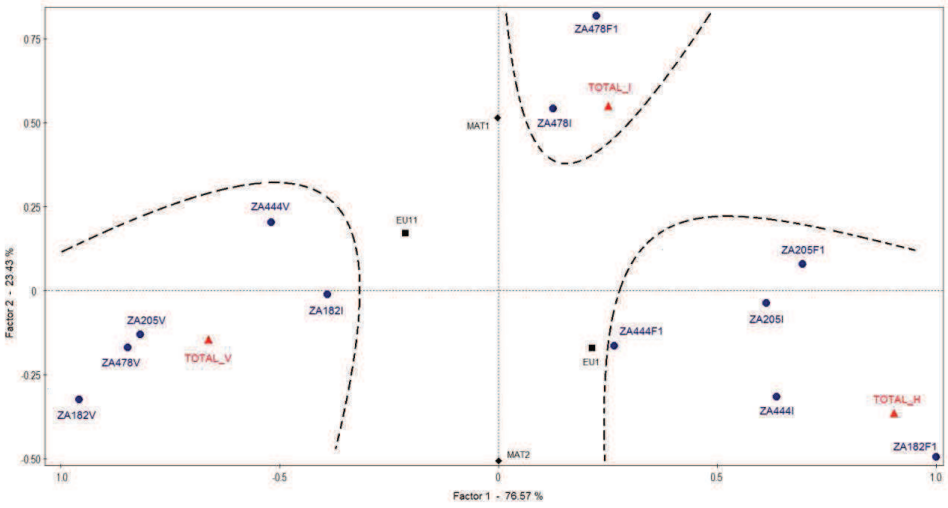
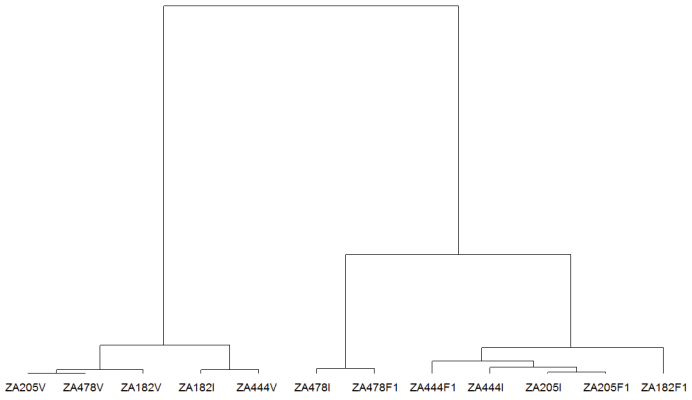


Figure 5.- Cluster analysis graph for classifying isolates depending on the first two coordinates from the factorial axes.



# Artículo original VI

Zamora, P.; Martín, A. B.; San Martín, R.; Martínez-Álvarez, P., Diez, J. J. 2014. Control of chestnut blight by the use of hypovirulent strains of the fungus *Cryphonectria parasitica* in northwestern Spain. Biological Control 79:58-66.

***Resumen***

Control del chancro del castaño mediante el empleo de aislados hipovirulentos del hongo *Cryphonectria parasitica* en el noroeste de España.

En Europa el chancro del castaño se controla mediante el empleo de *Cryphonectria hipovirus* CHV1, un virus de ARN no encapsulado. El hongo del chancro del castaño, *Cryphonectria parasitica*, se debilita por el virus y se produce un crecimiento de tejido sano del árbol hospedante. La transmisión de este hipovirus citoplasmático está restringida por un sistema de incompatibilidad del hongo, de manera que el hipovirus se puede transmitir únicamente entre aislados del mismo o parecido tipo de compatibilidad vegetativa (cv). Los aislados hipovirulentos de *C. parasitica* de Castilla y León, noroeste de España, (todos del subtipo francés CHV1-F1) fueron comparados con aislados virulentos tanto en laboratorio (sobre ramas) como en inoculaciones de campo (en dos sotos de la provincia de León y uno en la provincia de Zamora). Los test se llevaron a cabo con los tipos de cv más comunes en la región, EU1 y EU11. El ensayo en ramas reveló que los aislados hipovirulentos del tipo de cv EU1 no redujeron el crecimiento de los chancros virulentos. Por el contrario, cuatro aislados, H1, H4, H5 y H6 (todos del tipo de cv EU11) redujeron el crecimiento de los aislados virulentos en el ensayo de ramas. El test de campo mostró que los aislados hipovirulentos de EU1 y EU11 eran efectivos reduciendo el chancro en ambos sotos de León con todos los tratamientos utilizados, sin embargo, en Zamora, donde solo se testó EU11, todos los tratamientos fallaron excepto H1, que redujo el crecimiento del chancro al cabo de dieciocho meses tras la inoculación. El desarrollo de la hipovirulencia sugiere que el hipovirus del subtipo francés F1 está bien adaptado en la provincia de León. Tanto el hipovirus introducido de forma natural como el inoculado parecen haber reducido la incidencia del chancro mejorando con ello las masas de castaño. Sin embargo las inoculaciones no fueron tan efectivas en los sotos de Zamora. Esto indica que la enfermedad se podría controlar en Castilla y León mediante la inoculación de árboles con aislados hipovirulentos, pero se deberían hacer más pruebas en las provincias donde el hipovirus aún no está presente.

Biological Control 79 (2014) 58–66

Contents lists available at ScienceDirect

Biological Control

journal homepage: www.elsevier.com/locate/ybcon

# Control of chestnut blight by the use of hypovirulent strains of the fungus *Cryphonectria parasitica* in northwestern Spain

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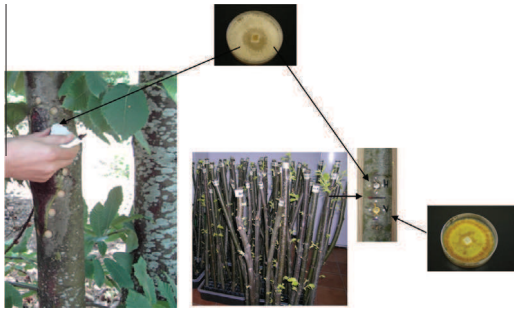
## HIGHLIGHTS

- Hypovirulent isolates of EU11 reduced canker lesions in dormant chestnut stems.
- Hypovirulent isolates of EU1 had no effect reducing canker lesions in the stems assay.
- Effective hypovirulent treatment of chestnut blight in León orchards.
- In Zamora orchards one of the three treatments had effect reducing canker growth.

## GRAPHICAL ABSTRACT

Field and cut stems inoculation with hypovirulent isolates from Castilla y León corresponding to the hypovirus subtype CHV1-F1.



## ARTICLE INFO

*Article history:*
Received 1 June 2014
Accepted 18 August 2014
Available online 26 August 2014

*Keywords:*
*Cryphonectria parasitica*
Hypovirus
Chestnut blight
Field inoculations

## ABSTRACT

Chestnut blight is controlled in Europe by using *Cryphonectria hypovirus* CHV1, a non-encapsulated RNA virus. The chestnut blight fungus, *Cryphonectria parasitica*, is weakened by the virus, and healing tissue growth occurs in the host tree. Transmission of this cytoplasmic hypovirus is restricted by the incompatibility system of the fungus, so that the hypovirus can be transmitted only between isolates of the same or closely related vegetative compatibility (vc) types. Hypovirulent isolates of *C. parasitica* (all of the French subtype CHV1-F1) from Castilla y León (NW Spain) were compared with virulent isolates in both laboratory (cut stems) and field inoculations (in two orchards in the province of León and one orchard in the province of Zamora). The tests were performed with the most common vc types in the region, EU1 and EU11. The cut stem assay revealed that the hypovirulent isolates of vc type EU1 did not reduce the growth of virulent cankers. By contrast, four hypovirulent strains H1, H4, H5 and H6 (all vc type EU11) reduced the growth of virulent isolates in the cut stem assay. Field tests showed that hypovirulent isolates of EU1 and EU11 were effective in reducing canker in both orchards in León with all treatments tested; however, in Zamora, where only EU11 was tested, all the treatments failed except H1, which was able to reduce growth of the canker eighteen months after the inoculation. The development of hypovirulence suggests that hypovirus subtype F1 is well adapted in the province of León. Both naturally extended and inoculated hypoviruses appear to have reduced the incidence of the canker, thus improving

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http://dx.doi.org/10.1016/j.biocontrol.2014.08.005

chestnut stands. However, the inoculations were not as effective in the orchards in Zamora. This indicates that the disease could be controlled in Castilla y León by inoculation of trees with hypovirulent strains, but that more tests should be done in provinces where the hypovirus is still not present.



## 1. Introduction

*Cryphonectria parasitica* (Murr.) Barr. is a fungal pathogen of *Castanea* and *Quercus* species (Heiniger and Rigling, 1994). Many years after the introduction of this pathogen in Europe, the fungus had reduced virulence in *Castanea sativa* Mill. and several chestnut populations have since been recovering (Robin and Heiniger, 2001; Turchetti et al., 2008), although it continues to be virulent in some areas. Decline of the disease is due to transmissible hypovirulence, a viral disease of the pathogen that reduces the virulence of the host fungus (Hillman and Suzuki, 2004; Prospero et al., 2006; Papazova-Anakieva et al., 2008). The virus is a non-encapsulated RNA virus of the genus *Hypovirus* that can be transmitted from infected to uninfected strains through hyphal anastomosis and also can move into fungal conidia (Ding et al., 2007; Prospero et al., 2006; Hogan and Griffin, 2008; Papazova-Anakieva et al., 2008). The hypovirulence trait has been used in Europe for the biological control of chestnut blight disease through the release of *Cryphonectria* hypovirus (CHV-1) (Heiniger and Rigling, 1994; Robin and Heiniger, 2001). The CHV-1 hypovirus is the most widely studied member of the family *Hypoviridae*, and five different subtypes have been identified. The subtypes known as subtypes F1 and F2 are of French origin, while subtype I is from Italy, subtype D is from Germany and subtype E is from Spain (Allemann et al., 1999).

Biological control of chestnut blight carried out in Slovakia with French hypoviruses (INRA Clermont-Ferrand) has moderated the incidence of the pathogen, thus increasing the health of the trees (Juhásová et al., 2005). In Italy, some coppices inoculated with hypovirulent strains were monitored during fifteen years and displayed a very low level of mortality due to chestnut blight as well as natural spread and persistence of hypovirulence throughout the treatment (Turchetti et al., 2008). The fitness of three CHV-1 subtypes (F1 and F2 and subtype I) were analysed in a French study, revealing that the Italian CHV-1 subtype I grew at similar rates and displayed similar sporulation levels as virus-free strains, in contrast to CHV1 subtypes F1 and F2, which greatly reduced the growth and sporulation of *C. parasitica* (Robin et al., 2010). The higher level of sporulation of subtype I make this subtype more invasive than subtypes F1 and F2. The first biological control assay with the Italian CHV-1 subtype I in Cataluña (Spain) yielded good results as regards reducing tree mortality. Almost all inoculated trees healed, and dispersion of the hypovirus was very high in both treated and untreated areas (Colinas et al., 2009). Other studies conducted with local hypoviruses belonging to CHV-1 showed that naturally occurring hypoviruses are potentially useful for biological control. This is the case in Macedonia, where five isolates of CHV-1 were analysed and yielded good results in slowing down canker development and tree mortality, as also found in other European studies using this technique (Sotirowski et al., 2011).

Transmission of the CHV between fungal individuals is restricted by their vegetative compatibility (vc) (Milgroom and Cortesi, 2004; Prospero et al., 2006; Papazova-Anakieva et al., 2008). Hypoviruses can be freely transmitted between individuals of the same vc type, but transmission between incompatible individuals depends on the genotypes of the interacting host individuals (Cortesi et al., 2001).

Another important aspect that controls the spread of the hypovirus is sexual compatibility, which is controlled by two mating type alleles (MAT-1 and MAT-2) at a single locus (Marra and Milgroom, 2001). The presence of both mating types in *C. parasitica* favours an increase in vc types by sexual reproduction, specifically via recombination of polymorphic *vic* genes (Cortesi and Migroom, 1998). Hypovirulence treatments are not effective in sexually reproducing *C. parasitica* because the sexual ascospores are always free of hypovirus (Carbone et al., 2004; Prospero et al., 2006).

Chestnut blight seriously affects orchards in the region of Castilla y León (NW Spain), where a total of eleven different vc types have been isolated; two of these (EU1 and EU11) are widely distributed throughout the region (Zamora et al., 2012). According to the mating type distribution, two of the provinces where *C. parasitica* appears (León and Ávila) are mainly affected by MAT-1, and the other two provinces (Zamora and Salamanca) are affected by both mating types (Zamora et al., 2012). Within Castilla y León, the provinces of León and Zamora have the largest blight-affected chestnut populations in the region, whereas *C. parasitica* is just beginning to spread in Salamanca and Ávila.

Fifteen isolates containing dsRNA were detected in the province of León (Montenegro et al., 2008): fourteen isolates of the vc type EU1 and one of the unknown vc type E3, which was not compatible with the 74 European vc types. Fourteen new hypovirus-infected *C. parasitica* isolates have recently been identified (Zamora et al., 2012): nine in vc type EU1 and five in EU11. All isolates analysed contained the French hypovirus CHV-1-subtype F1 (Montenegro et al., 2008; Zamora et al., 2012). The existence of natural hypovirulence, the vc type and mating type distribution enables the application of biological control in the region.

The aims of this study were to evaluate the effectiveness of regional isolates containing hypovirus for the biological control of chestnut blight in different chestnut stands in Castilla y León and to select suitable hypoviruses for infecting EU1 and EU11, which are the most frequent vc types of the fungus in the region.

## 2. Materials and methods

### 2.1. Field plots

Field experiments were performed in different orchards: Robledo, in the province of Zamora, and Médulas and Berlanga del Bierzo, in the province of León. The meteorological characteristics of the plots (rainfall in mm; minimum, mean and maximum temperature in °C) were 1062.1 mm, 5.5 °C, 11 °C and 16.5 °C in Berlanga del Bierzo, 912.9 mm, 4.7 °C, 10.3 °C and 16 °C in Médulas and 995 mm, 4.1 °C, 10.1 °C and 16.2 °C in Robledo (Ninyerola et al., 2005).

### 2.2. Vegetative compatibility types and mating types

A representative number of cankers were chosen at random and sampled in each plot (one isolate per canker, Table 1). The samples were cultured on potato dextrose agar (PDA Difco) in the laboratory at 25 °C and analysed for orange/white pigmentation and corresponding vc type and mating type. The barrage/merging response was used for identification of vc type (Anagnostakis et al., 1986), and the cultivation medium was PDAg (Powell,

**Table 1**
Number of isolates of *Cryphonectria parasitica* from each selected area and the distribution of VC and mating types.

| Province | Location | No of isolates | vc type | | | | | | | MAT-1 | MAT-2 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | EU1 | EU11 | EU12 | EU66 | CL4 | CL10 | No *C. parasitica* | | |
| León | Médulas | 261 | 81 | 167 | 1 | | 1 | | 11 | 150 | |
| León | Berlanga | 52 | 50 | | | | | | 2 | 50 | |
| Zamora | Robledo | 231 | 10 | 195 | 3 | 1 | | 7 | 7 | 10 | 40 |

1995 modified): 24 g Difco Potato Dextrose Agar, 2 g yeast extract, 7 g malt extract and 0.8 g tannic acid adding bromocresol green (50 mg $l^{-1}$) to enhance the visualization of incompatible reactions (Cortesi et al., 1998). The first confrontations were made with the collection of vc type testers from Castilla y León obtained in a previous work (Zamora et al., 2012). This collection includes isolates from vc types coincident with the European vc testers EU1 to EU74 kindly provided by Cortesi and Migroom (1998) and Robin et al. (2000). Those that were not coincident with the European tester are called with the letters CL and a number. The mating type was analysed by PCR amplification, as described by Zamora et al. (2012), with the primers M1-GS1 and M1-GS2-rev for MAT-1 and primers M2-GS2 and InvA5n for MAT-2 (Marra and Milgroom, 1999; McGuire et al., 2001). This analysis was performed to prevent the introduction of new vc types or mating types with the hypovirulent inoculum. The white isolates were analyzed for dsRNA and the orange isolates were selected as candidates for the inoculation assays.

### 2.3. Hypovirulent isolates

Previous analysis of a partial sequence of the viral genome isolates revealed a group of nine viral isolates with an identical sequence. This group included isolates LE12 (from Viariz, León), LE171, LE172 and LE182 (from Orellán, León) used as donors in the present study. The donor isolate LE64 (Corullón, León) differed from this group by only two nucleotide sequences (Zamora et al., 2012). The phylogenetic tree showed that the viral isolates grouped with CHV-1-EP713 (the reference hypovirus of subtype F1), and were clearly distinct from all other CHV-1 subtypes (Zamora et al., 2012).

Some isolates were previously transformed to hypovirulence by horizontal transmission of the hypovirus in the laboratory (Tables 2 and 3). This was done to transform some isolates used in cut stems and field assays (ZA 182, ZA130, ZA54, LE182, LE103, LE410 and LE416) (Tables 2 and 3). Transformations were performed to prevent introduction of new strains of *C. parasitica* into the inoculation areas. Horizontal transmission of the hypovirus in the isolates previously transformed in the laboratory was conducted by co-culturing a virulent (V) with a hypovirulent (H) isolate (5 mm apart) in a Petri dish containing PDA (Difco). The isolates were cultured for 10 days at 25 °C in darkness. All pairs of H and V isolates belonged to the same vegetative compatibility type, and the trials were replicated five times with each pairing. Successful transformation of the V isolates into H was detected by the presence of mycelial sectors with the white appearance characteristic of the hypovirulent phenotype. These sectors were isolated and analysed for hypovirulence by the CF-11 cellulose purification procedure (Morris and Dodds, 1979; Rigling et al., 1989). At the end of this procedure, white *C. parasitica* strains containing hypovirus dsRNA were considered hypovirulent. The growth rate of the resulting hypovirulent isolates (on PDA) was not significantly different from that of the wild strains. Wild strains H5 (LE172) and H7 (LE64) and the transformed H2 (LE172+ ZA182) and H10 (LE64+ ZA54) grew more slowly than all the other isolates but all of the hypovirulent isolates grew faster than the virulent isolates (data not shown). Pigmentation information about the hypovirulent isolates is given in Table 3.

### 2.4. Hypovirulent inoculum

The selected hypovirulent strains were grown in PDA (Difco) for seven days at 25 °C. On average, 10 colonies from each selected isolate were grown and ground to a paste, with an electric mixer, under sterile conditions. The paste was mixed with one litre of PDA and after seven days at 25 °C dispensed into sterile aluminium

**Table 2**
Hypovirulent and virulent isolates used in the laboratory inoculations of cut stems.

| Isolate code[a] | Vc type | Mating type | dsRNA[b] | Isolate pigmentation | Isolate name[c] | Province | Location |
|---|---|---|---|---|---|---|---|
| H1 | EU11 | MAT-2 | + | Pale orange | LE171 + ZA182 (a) | Zamora | Robledo |
| H2 | EU11 | MAT-2 | + | Pale orange | LE172 + ZA182 (a) | Zamora | Robledo |
| H3 | EU11 | MAT-2 | + | White | LE182 + ZA182 (a) | Zamora | Robledo |
| H4 | EU11 | MAT-1 | + | White | LE171 | León | Médulas |
| H5 | EU11 | MAT-1 | + | Pale orange | LE172 | León | Médulas |
| H6 | EU11 | MAT-1 | + | White | LE182 | León | Médulas |
| H7 | EU1 | MAT-1 | + | White | LE64 | León | Berlanga |
| H8 | EU1 | MAT-1 | + | Pale orange | LE 12 | León | Corullón |
| H9 | EU1 | MAT-1 | + | Pale orange | LE12+ZA130(a) | Zamora | Robledo |
| H10 | EU1 | MAT-1 | + | White | LE64+ZA54(a) | Zamora | Robledo |
| V1 | EU11 | MAT-1 | – | Orange | ZA13 (b) | Zamora | Robledo |
| V2 | EU11 | MAT-2 | – | Orange | ZA182 (b) | Zamora | Robledo |
| V3 | EU1 | MAT-1 | – | Orange | ZA11(b) | Zamora | Robledo |
| V4 | EU1 | MAT-1 | – | Orange | ZA54(b) | Zamora | Robledo |
| V5 | EU1 | MAT-1 | – | Orange | ZA130(b) | Zamora | Robledo |

[a] Identification code for the isolates used in the inoculation assay: H hypovirulent isolates, V virulent isolates.
[b] Hypovirulent isolates containing dsRNA (+) and virulent isolates without dsRNA (–).
[c] Names of isolates: designated name for the isolates in the *Cryphonectria parasitica* collection from Castilla y León, maintained in the Centro de Sanidad Forestal de Calabazanos. (a) Hypovirulent isolates previously transformed in the laboratory with isolates LE171, LE172, LE182, LE12 and LE64 (of field origin) (Zamora et al., 2012) as hypovirulent donors and (b) ZA13, ZA 182 and ZA11, ZA130, ZA54 as virulent receptors for vc type EU11 and vc type EU1 respectively.

**Table 3**
Characteristics and pigmentation of hypovirulent *Cryphonectria parasitica* isolates used in the field inoculations.

| Isolate[a] | Vc type | Mating type | Isolate pigmentation | Isolate name[b] | Province | Location |
|---|---|---|---|---|---|---|
| H1 | EU11 | MAT-2 | Pale orange | LE171 + ZA182 (a) | Zamora | Robledo |
| H2 | EU11 | MAT-2 | Pale orange | LE172 + ZA182 (a) | Zamora | Robledo |
| H3 | EU11 | MAT-2 | White | LE182 + ZA182 (a) | Zamora | Robledo |
| H4 | EU11 | MAT-1 | White | LE171 | León | Médulas |
| H5 | EU11 | MAT-1 | Pale orange | LE172 | León | Médulas |
| H11 | EU11 | MAT-1 | Pale orange | LE171 + LE103 (a) | León | Médulas |
| H12 | EU11 | MAT-1 | Pale orange | LE172 + LE103 (a) | León | Médulas |
| H13 | EU1 | MAT-1 | White | LE64 + LE410 (a) | León | Berlanga |
| H14 | EU1 | MAT-1 | White | LE64 + LE416 (a) | León | Berlanga |

[a] Identification code of the isolates used in the inoculation assay: H hypovirulent isolates.
[b] Names of isolates: designated name for the isolates in the *Cryphonectria parasitica* collection from Castilla y León, maintained in the Centro de Sanidad Forestal de Calabazanos. (a) Hypovirulent isolates previously transformed in the laboratory with isolates LE171, LE172, LE182 and LE64 (of field origin) (Zamora et al., 2012) as hypovirulent donors.

tubes and reserved at 4 °C until the inoculation. PDA without inoculum was dispensed into tubes for use as controls.

### 2.5. Cut stem inoculation assay in the laboratory

Stems of dormant *Castanea sativa* (130 cm long and 3–5 cm wide), from areas free of the disease, were inoculated in the laboratory with hypovirulent and virulent strains of *C. parasitica*. In total, 10 isolates containing dsRNA and five dsRNA free isolates (V1 = ZA13, V2 = ZA182, V3 = ZA11, V4 = ZA54 and V5 = ZA130) were used to inoculate 65 chestnut stems (Table 2). The hypovirulent (H) and virulent (V) isolates were inoculated alone and in strain pairings (H + V) with isolates of the same vc type. The pairings were inoculated with a distance of 2 cm between them. Two stems with three inoculations were used for each isolate or pairing. Successive pairs were inoculated at an angle of 90° from each another in the stem. This was done to prevent necrotic lesions between two adjacent inoculations merging. Five millimeter (Ø) plugs of bark plus tissue, including the vascular cambium, were removed with a cork borer. Mycelia of *C. parasitica* were placed in the resulting holes, and the inoculated stems were sealed with Parafilm to minimize desiccation. The stems were kept in a dark room at 25 °C with the basal end of the stems in water. The surface area of the canker lesion was estimated after inoculation by applying the ellipse area formula (Elliston, 1978; Turchetti and Maressi, 1991) to the lengths and widths measured at ten-day intervals during one month. When the virulent isolates were placed next to the hypovirulent isolates, the virulent canker lesion was measured to monitor any changes in growth.

### 2.6. Field tests

Test inoculations were conducted on cankers once the vc type and mating type of isolates were identified, and any that differed from the inocula were discarded. The hypovirulent isolates used were either obtained from orchards in the province of León or had previously been transformed in the laboratory (Table 3). As already mentioned, the transformations were carried out to prevent the introduction of new strains of *C. parasitica* in the inoculation areas. The selected cankers were easily accessible and the lesion had a well defined margin. The diameters of the cankers (length and width) were measured before the inoculation. The inoculation was performed by first punching a line of holes (5 mm Ø) separated 4 cm from each other in the cankers. These holes were filled with the hypovirulent inoculum of *C. parasitica* selected for each zone and were then covered with plastic tape (Fig. 1). Control cankers were inoculated with sterile ground PDA (Difco). The inoculation intensity was 6 trees per hectare. The total number of hectares inoculated in each treatment was 9 ha (54 trees) in Médulas, 6 ha (35 trees) in Berlanga del Bierzo and 4 ha (25 trees) in Robledo. Cankers of vc type EU11 were used for inoculations in Médulas and Robledo, and vc type EU1 was used in Berlanga del Bierzo (Table 3). The effect of hypovirulence was evaluated (as growth of cankers) 6 and 12/18 months later. Canker length and width were measured in each period and the surface area of each canker was calculated using the formula for the area of an ellipse (Elliston, 1978; Turchetti and Maressi, 1991). The percentage increase in growth (PIG) was calculated from the increase in the canker area ($PGI = (Aj - A_i/A_i) * 100$) to analyse the effect of the inoculations over time. After a period of two years, some cankers from each of the inoculation areas were resampled and analysed for the presence of dsRNA.

### 2.7. Data analysis

We used a mixed model to examine whether the hypovirulent isolates reduced the growth (area) of the virulent cankers in the laboratory inoculated cut stems. The model is represented by the following equation: $y_{ijkn} = \mu + VCG_i + T_j + VCG_i \times T_j + I_k(VCG_i \times T_j) + \xi_{ijkn}$, where $\mu$ is the general mean, $VCG_i$ is the effect of the vegetative compatibility type (EU1, and EU11), $T_j$ represents the different treatment (hypovirulent, virulent or hypovirulent + virulent), $VCG_i \times T_j$ is the interaction between both factors, $I_k$ is the effect of the different isolates used in each treatment and in each vegetative compatibility type, and $\xi_{ijkn}$ is the error terminus of the model. Errors were normally distributed and independent; REML (Restricted Maximum Likelihood) variances were calculated for all combinations of VCG and treatment. The response variable, $y_{ijnk}$, was transformed by *ln* (1 + Area) where Area is the surface area of the canker calculated using the ellipse formula (Elliston, 1978; Turchetti and Maressi, 1991). This transformation was necessary to ensure the normality of the random error terminus.

To check the main hypothesis that the hypovirulent inoculated cankers reduce growth of the canker lesions in the field assay, we used a repeated measures model to analyse the data from each of the three assays (Berlanga del Bierzo, Médulas and Robledo) $y_{in:j} = \mu + I_i + t_j + I_i \times T_j + \xi_{in:j}$ where μ is the general mean, $I_i$ is the effect of isolate, $T_j$ is the effect of the measurement time period, $I_i \times T_j$ is the interaction between isolate and measuring time period, and $\xi_{in:j}$ is the random error of the model. Errors were normally distributed with zero mean, constant variance for each time of measure, independent for different isolates and with fixed covariance for different measuring times in the same isolate. REML (restricted maximum likelihood) variances were calculated. The response variable, $y_{in:j}$ was transformed by *ln* (1 + PIG), where PIG is the percentage increase in growth ($PIG = (A_j - A_i/A_i) * 100$), and A is the surface area calculated using the ellipse formula


**Fig. 1.** Field inoculation procedure. A: Making the line of holes surrounding the canker. B,C: Filling the holes with the inoculum. D: Covering holes with plastic tape.

(Elliston, 1978; Turchetti and Maressi, 1991). This transformation was necessary to ensure the normality of the random error terminus.

Orthogonal contrasts and LSD tests were used to detect significant differences.

## 3. Results

### 3.1. Vc type and mating type analysis

The most abundant vc types in León and Zamora were EU1 and EU11. In León, another two strains were isolated, one from vc type EU12 and one from vc type CL4, whereas other vc types were also present in Zamora (EU12, EU66, CL10), EU11 was the most abundant. Regarding the mating type distribution, only MAT-1 occurred in León, while both mating types (MAT-1 and MAT-2) were present in Zamora, with MAT-2 being the most abundant (Table 1).

### 3.2. Cut stem inoculation assay

The inoculations with vc type EU1 did not show any differences between hypovirulent, virulent and the combination of both, virulent with hypovirulent isolates ($p > 0.05$) (Fig. 2). In contrast, differences were observed with vc type EU11 when the hypovirulent and virulent isolates were inoculated together, reducing the growth of the virulent cankers ($p < 0.05$) (Fig. 2). All hypovirulent isolates from vc type EU1 displayed similar growth, with the exception of H10 ($p < 0.05$), as did the virulent isolates (Fig. 3). In contrast, hypovirulent and virulent isolates of vc type EU11 differed from each other (Fig. 3). Regarding vc type EU11, hypovirulent isolates H2 and H3 did not decrease the growth of both virulent isolates tested (V1 and V2) ($p > 0.05$). In contrast, hypovirulent isolates H1, H4 and H6 reduced the growth of the virulent isolate V2, and H5 reduced the growth of isolate V1 (Fig. 4).

### 3.3. Effect of hypovirulent isolates in the field

The inoculations in the Robledo orchard (province of Zamora) did not show any differences either between treatments or between treatments and the control ($p > 0.05$). However, after eighteen months treatment, H1 decreased growth of the canker relative to that of the control ($p = 0.05$) (Fig. 5). After a period of two years, the molecular analysis revealed the presence of dsRNA in only one of the 31 cankers analysed (one sample per canker, from inoculated cankers).

In Berlanga del Bierzo (province of León) growth of the whole cankers was similar six months after inoculation ($p_{H13\text{-}Tc} = 0.88$; $p_{H14\text{-}Tc} = 0.33$). Eighteen months later, differences were observed relative to the control ($p_{H13\text{-}Tc} = 0.03$; $p_{H14\text{-}Tc} = 0.001$), with no difference between the two H isolates tested ($p_{H13\text{-}H14} = 0.08$) (Fig. 5). Double-stranded RNA was detected after two years in five of fourteen isolates.

Six months after the inoculation of EU11 isolates in Médulas (province of León), growth of all the cankers was very similar. However, the inoculated canker tended to grow less than the controls (TC). Twelve months after inoculation of the hypovirulent strains, the size of the treated canker did not increase relative to the control lesions ($p_{H4\text{-}TC} = 0.01$; $p_{H5\text{-}TC} = 0.02$; $p_{H11\text{-}TC} = 0.01$;

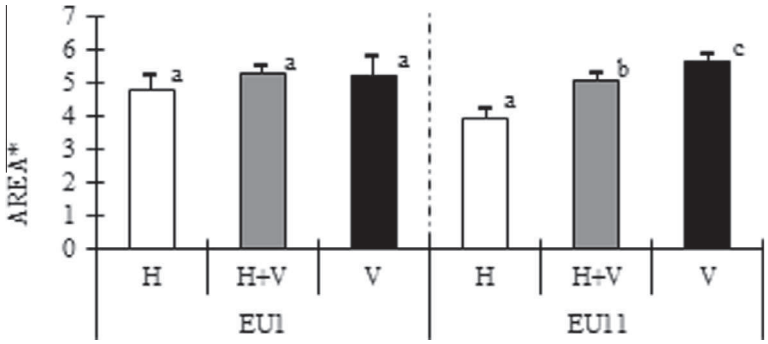


**Fig. 2.** Canker lesion area, in cut stems inoculated in the laboratory, caused by the virulent and hypovirulent strains and the combination of both strains of vc types EU1 and EU11 one month after inoculation. Black bars represent the median growth of the virulent (V) isolates, grey bars represent the paired isolates (H + V) and white bars represent the hypovirulent isolates (H). Error bars represent 95% confidence intervals. Letters a, b and c indicate statistically significant differences, relative to EU11. EU11 had no significant differences between treatments. *Area = Ln (1 + canker area ($cm^2$)). The area was calculated with the ellipse formula (Elliston, 1978; Turchetti and Maressi, 1991).

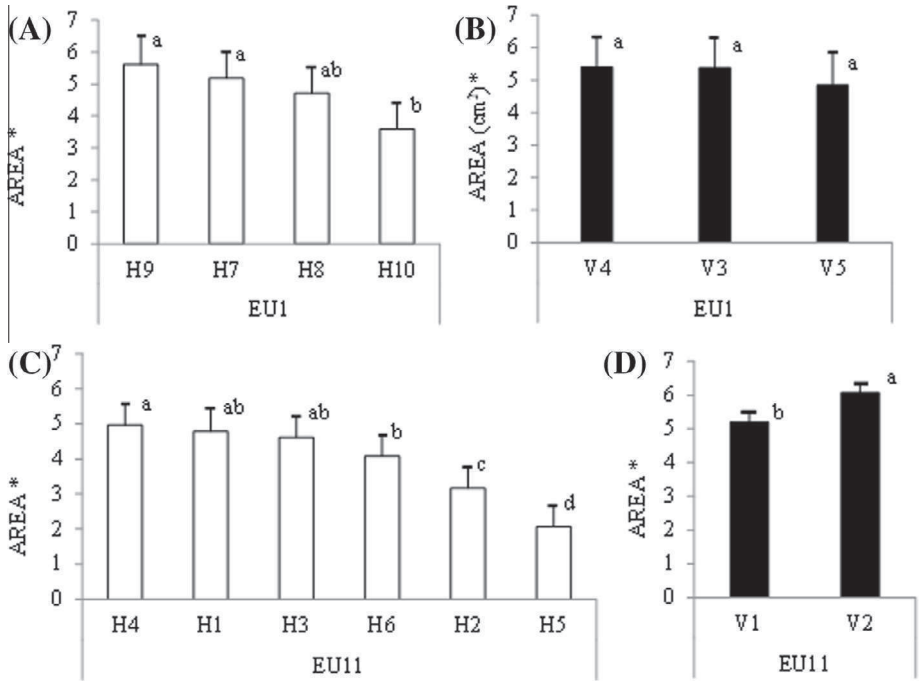


**Fig. 3.** Canker lesion area in cut stems inoculated in the laboratory caused by the hypovirulent (A) and virulent (B) strains of vc types EU1 and hypovirulent (C) and virulent (D) strains of vc type EU11 one month after inoculation. Error bars represent 95% confidence intervals. *Area = Ln (1 + canker area ($cm^2$)). The area was calculated with the ellipse formula (Elliston, 1978; Turchetti and Maressi, 1991).

$p_{H12\text{-}TC} = 0.01$) (Fig. 5). At the end of the assay, there were no differences between the treatments with the wild isolates (H4 and H5) and isolates transformed in the laboratory (H11 and H12) ($p > 0.1$). The cankers inoculated with hypovirulent isolates formed calluses and survived the infections. After two years, dsRNA was present in five (vc type EU11) of 25 isolates analysed.

## 4. Discussion

The field inoculations reduced the growth of almost all cankers. The treated cankers produced scar tissue and growth of the lesions was slowed. The effectiveness of the hypovirulence treatments differed among orchards and was more successful in León (Médulas and Berlanga del Bierzo) than in Zamora (Robledo). Successful transmission of hypovirulence may be possible in León because of the prior presence of hypovirulent strains of the most extended vc types (EU1 and EU11) and the low diversity resulting mainly from asexual reproduction (only MAT-1 present) (Anagnostakis et al., 1986; Liu et al., 2000; Cortesi et al., 2001; Papazova-Anakieva et al., 2008; Sotirowski et al., 2011). Conversely, no natural hypovirulence has been observed so far in Zamora. In 2011, various analyses carried out by the regional government of Castilla y León in the orchards in Zamora indicated that hypovirulence did not occur naturally in the trees. The lack of hypovirulent strains together with the high diversity of strains in Zamora (7 different vc types and two mating types: Zamora et al., 2012) hinders the transmission of hypovirulence.

The hypovirulent strains detected in Castilla y León contained the CHV1 subtype F1 (Zamora et al., 2012; Montenegro et al., 2008). Previous studies in France showed that French hypovirulent strains of *C. parasitica* containing the CHV1 subtype F1 caused very small lesions and few stromata (Robin et al., 2010). In the laboratory assay, when the cut stems were inoculated with hypovirulent strains from Castilla y León containing hypovirus CHV1 subtype F1, in most cases the growth was similar to the growth of the virulent

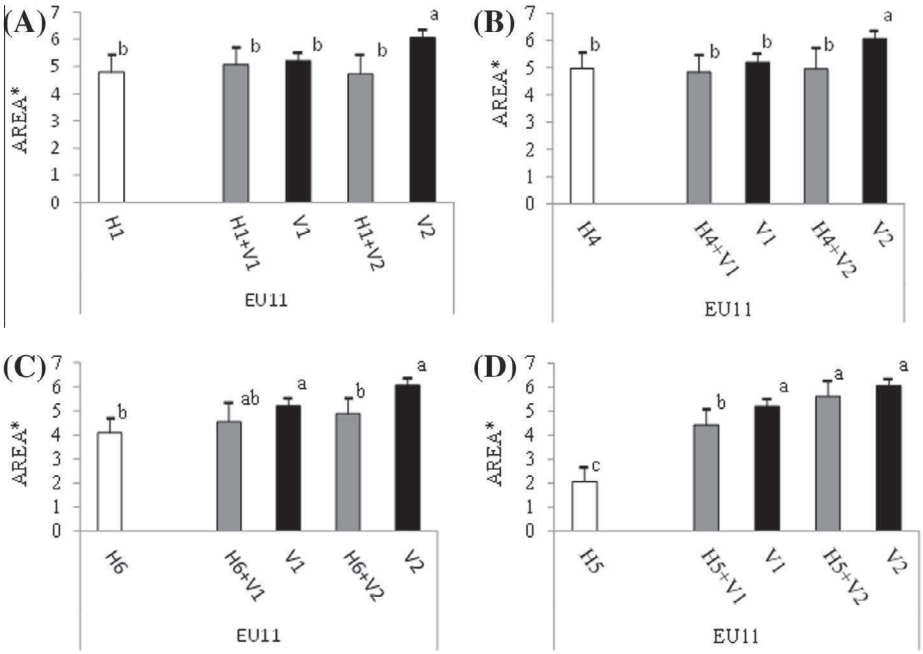


**Fig. 4.** Canker lesion area in cut stems caused by hypovirulent isolates H and combination of this with the virulent isolates (H + V1, H + V2) and compared with the virulent isolates (V1 and V2) in vc type EU11. (A) Results of hypovirulent isolate H1, (B) results of hypovirulent isolate H4, (C) results of hypovirulent isolate H6 and (D) results of hypovirulent isolate H5. Black bars represent shows the median growth of the virulent (V) isolates, grey bars represent the combination of isolates (H + V) and white bars represent the hypovirulent isolates (H). Error bars represent 95% confidence intervals. *Area = Ln (1 + canker area ($cm^2$)). The area was calculated with the ellipse formula (Elliston, 1978; Turchetti and Maressi, 1991).

strains, contrary to expectations. The different behaviour of the CHV-1 subtype F1 may be due to interactions between host, parasite and the environment in the chestnut blight pathosystem, as demonstrated in a previous study (Bryner and Rigling, 2011). The similar growth of the hypovirulent and virulent strains in EU11 was not as evident (only strains H2 and H3 grew similar to the virulent strains) and resembled the pattern observed in France (Robin et al., 2010). In addition, when horizontal transmission of hypovirus from Castilla y León strains was tested on PDA media, the strains of EU1 and hypovirus CHV1-F1 were more effective than EU11 strains (data not shown). Some of the hypovirulent strains previously transformed in the laboratory behaved differently, even with the same hypovirus. This occurred with isolates H5 and H6 (field origin) and H2 and H3 (previously transformed in the laboratory) (all vc type EU11); the wild hypovirulent isolates (H5, H6) showed some response to *C. parasitica* in the cut stem inoculations unlike the previously transformed isolates (H2, H3), which had no effect on canker growth. This reinforces the theory of the possible influence of the fungus in the behaviour of the hypovirus. Moreover, EU11 showed some effect with four isolates when co-cultured with the virulent isolates in the cut stems, three of field origin (H4, H5 and H6) and one which was transformed in the laboratory (H1). In the field, H1, H4 and H5 also successfully reduced canker growth. All of the isolates of vc type EU1 were effective in reducing canker growth in the field; however, in the cut stems assay no effect was observed. This fact could be due to the dormancy breakage of the stems at the end of the assay.

In the field treatments, the cankers inoculated with hypovirulent isolates continued growing quickly during the first six months, with no differences between these and the controls. However, one year after the inoculation, growth of the cankers had slowed down. Sotirowski et al. (2011) analysed the variation in virulence of CHV-1 in Macedonia during thirteen months after inoculating chestnut stems in the field and observed that the growth of the isolates infected with the CHV-1 began to decrease after eleven months. Other authors also observed lower growth rates of hypovirulent strains one year after inoculations (Ding et al., 2007). These results suggest that the effectiveness of chestnut blight control should be monitored for at least one year after inoculation.

Both vc types tested showed good results in reducing the canker growth in León – indicating that biological control with hypovirulence may be successful in the region. However, the treatment was not as effective in Zamora, because only one of the three treatments of vc type EU11 reduced the growth of the cankers (H1). Although more vc types are found in Zamora than in León and both mating types are present in the former, the inoculation was carried out in cankers with vc type EU11 because it was the only type that was abundant in Robledo. Thus, it is not clear whether the different behaviour of the hypovirus is linked to the vc type or to different environmental conditions. Although the *C. parasitica* populations differ between León and Zamora, the inoculation was always done with the same vc type in previously analyzed cankers. The only difference in the isolates used the inoculations of EU11 in León and Zamora was that those from León were of mating type MAT-1 and those from Zamora were MAT-2. Other studies indicate that in addition to the vic genes, the host genetic background also affects transmission of the virus (Cortesi et al., 2001). The heterokaryon incompatibility is also effective in reducing the spread of infectious elements (Smith and Milgroom, 2006). Future studies should examine whether the mating type has any influence in the horizontal transmission of the hypovirus between isolates in Castilla y León populations of *C. parasitica*, to clarify the difference in the behaviour of both populations (Zamora and León). The only isolate in Zamora with good results in halting growth of the canker in the laboratory and in the field was H1 (which was previously converted in the laboratory with H4). It is possible that the fungal isolate containing the hypovirus is more important than previously thought.

Two years after inoculation, recovery of strains with dsRNA from inoculated and non inoculated cankers indicates a low presence of hypovirulence in the field in León and negligible presence in Zamora. In León, the recovery of hypovirulent isolates was not

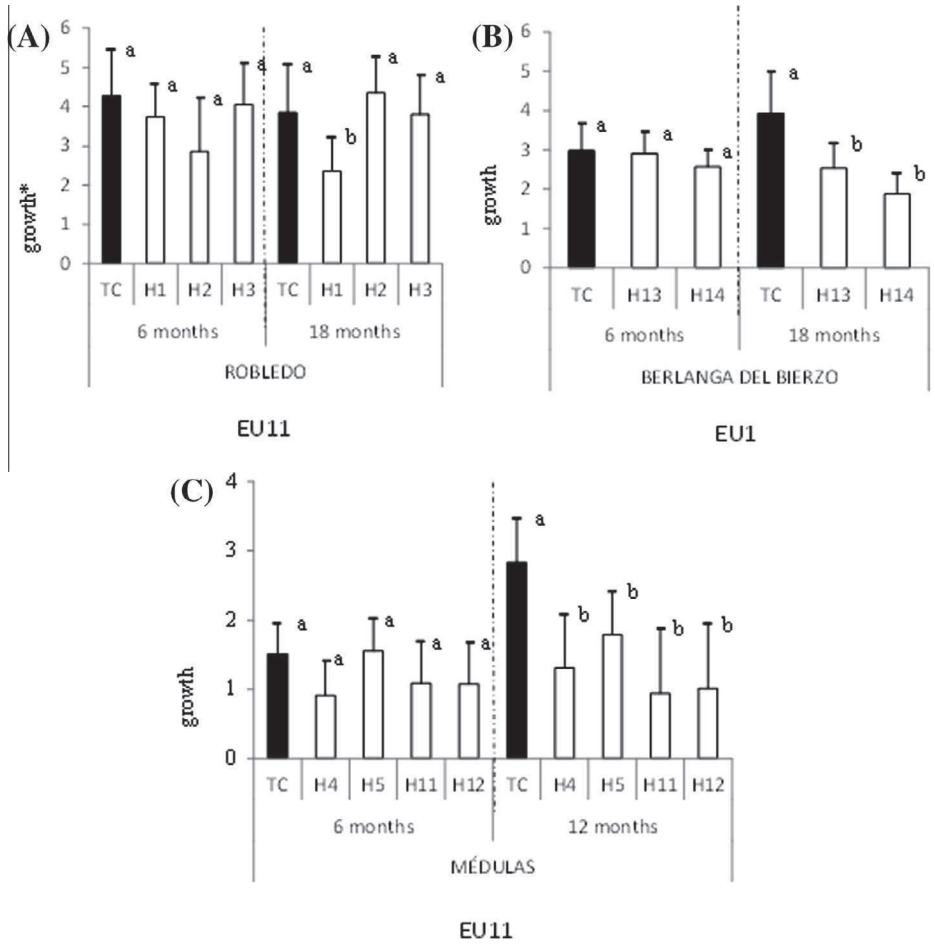


**Fig. 5.** Mean relative canker growth after treatment of inoculated (white bars) and non inoculated (black bars) cankers in Robledo (A), Berlanga del Bierzo (B) and Médulas (C) orchards after 6 and 12–18 months. Robledo and Médulas were inoculated with isolates of vc type EU11 and Berlanga del Bierzo was inoculated with isolates of vc type EU1. TC: control treatment, H1, H2, H3, H11, H12, H13, and H14: treatments with isolates previously transformed in the laboratory and H4 and H5: treatments with wild hypovirulent isolates. Error bars represent 95% confidence intervals. *Growth calculated with the formula Ln (1 + PIG), where (PIG = $(A_j - A_i/A_i) * 100$) and $A$ = area calculated with the ellipse formula.

very high but form both vc types EU1 and EU11. Only one hypovirulent isolate was recovered from an inoculated canker in Zamora. The low rate of presence of hypovirus was more marked in EU11 in both provinces. This suggests that the vc type might have some influence on the survival and dissemination of the hypovirus. Vc type EU11 is not a frequent type in the European chestnut blight distribution and the different behaviour perhaps has more to do with this concrete vc type than expected. Recent studies established molecular identities of genes associated with four *C. parasitica* vic loci which interact in the incompatibility system and restrict virus transmission (Choi et al., 2012). Perhaps these tightly linked genes have influence in the transmission of the hypovirus between isolates from vc type EU11.

It would be interesting to isolate new hypovirus strains in the inoculated areas during the next few years to observe the permanence of the hypovirus and to test the ecological fitness of the CHV-1-subtype F1 in the region. The lower growth rate and the reduction in the production of conidia in the strains containing hypovirus F1 make this CHV-1 subtype suitable for therapeutic control (Robin et al., 2010). The incidence of hypovirulence appears to be increasing in the province of León. However, so far all the isolates tested belong to the CHV-1 subtype F1. Together with the results of the inoculations done in the present study, this suggests that hypovirus CHV-1 subtype F1 may be well adapted to the dispersal in the region. So far, it seems that both naturally extended and inoculated hypoviruses have reduced the incidence of the canker in León, thus improving the chestnut stands.

This indicates that in Castilla y León, the disease may be controlled by hypovirulence, at least in those orchards or plantations with low vc type diversity; however, more tests must be done in provinces where the hypovirus is still not present.

## Acknowledgments

This research was supported by grants provided by the Regional Government of Castilla y León and the project "*Control biológico del chancro del castaño en Castilla y León*" VA053A08. The authors thank Eva Mayor for technical assistance and help with fieldwork.

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