



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.



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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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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.

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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 Migroo, 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		
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

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 3Characteristics 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 ($PGI = (A_j - A_i/A_i) * 100$) 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 μ 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 ξ_{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_{ijkn} , was transformed by $\ln(1 + \text{Area})$ 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) $y_{inj} = \mu + I_i + t_j + I_i \times T_j + \xi_{inj}$ 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 ξ_{inj} 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_{inj} was transformed by $\ln(1 + \text{PIG})$, 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 ($p_{H13-Tc} = 0.88$; $p_{H14-Tc} = 0.33$). Eighteen months later, differences were observed relative to the control ($p_{H13-Tc} = 0.03$; $p_{H14-Tc} = 0.001$), with no difference between the two H isolates tested ($p_{H13-H14} = 0.08$) (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 ($p_{H4-Tc} = 0.01$; $p_{H5-Tc} = 0.02$; $p_{H11-Tc} = 0.01$;

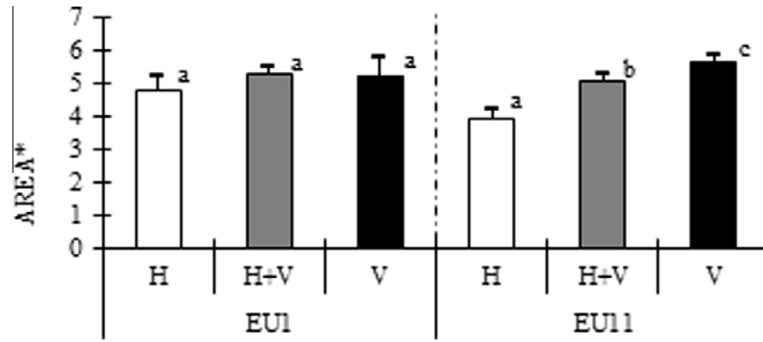


Fig. 2. Canker lesion area, in cut stems inoculated in the laboratory, caused by the virulent and hypovirulent strains and the combination of both strains of vc types EU1 and EU11 one month after inoculation. Black bars represent the median growth of the virulent (V) isolates, grey bars represent the 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 (cm²)). The area was calculated with the ellipse formula (Elliston, 1978; Turchetti and Maressi, 1991).

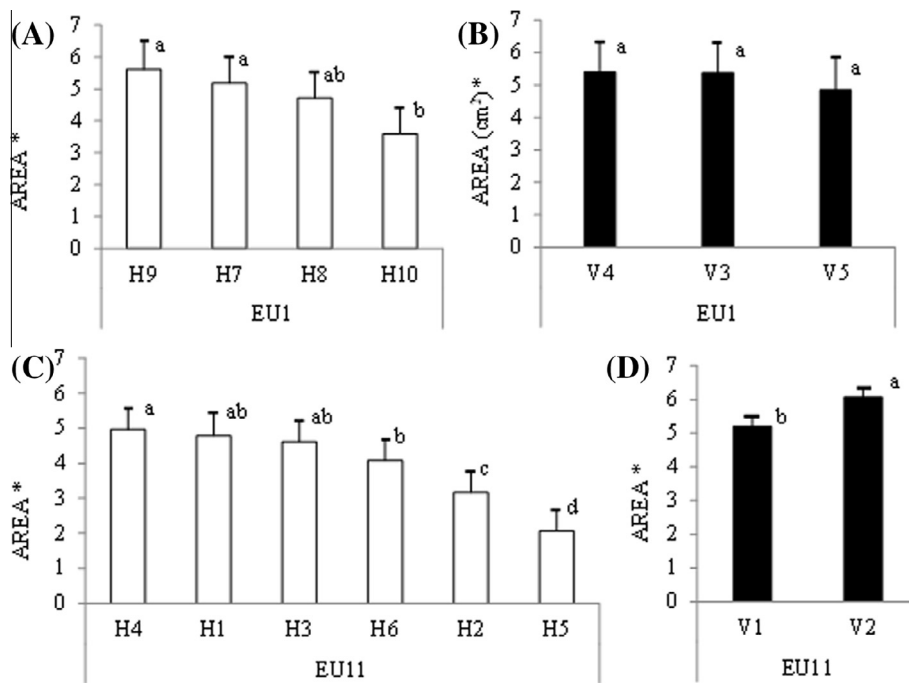


Fig. 3. Canker lesion area in cut stems inoculated in the laboratory caused by the hypovirulent (A) and virulent (B) strains of vc types EU1 and hypovirulent (C) and virulent (D) strains of vc type EU11 one month after inoculation. Error bars represent 95% confidence intervals. *Area = Ln (1 + canker area (cm²)). The area was calculated with the ellipse formula (Elliston, 1978; Turchetti and Maressi, 1991).

$p_{H12-TC} = 0.01$) (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

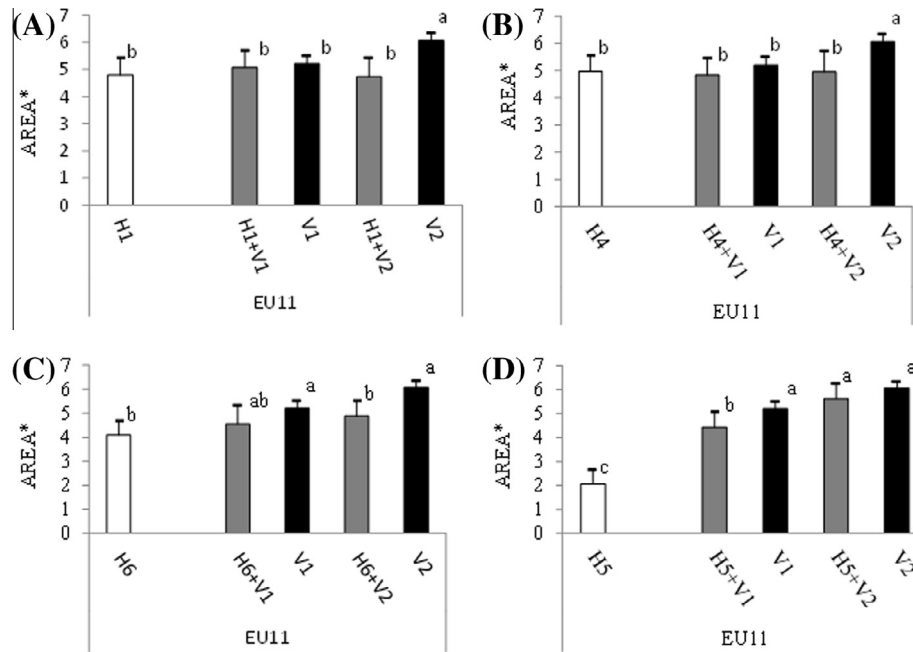


Fig. 4. Canker lesion area in cut stems caused by hypovirulent isolates H and combination of this with the virulent isolates (H + V1, H + V2) and compared with the virulent isolates (V1 and V2) in vc type EU11. (A) Results of hypovirulent isolate H1, (B) results of hypovirulent isolate H4, (C) results 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 = $\ln(1 + \text{canker area (cm}^2\text{)})$. 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

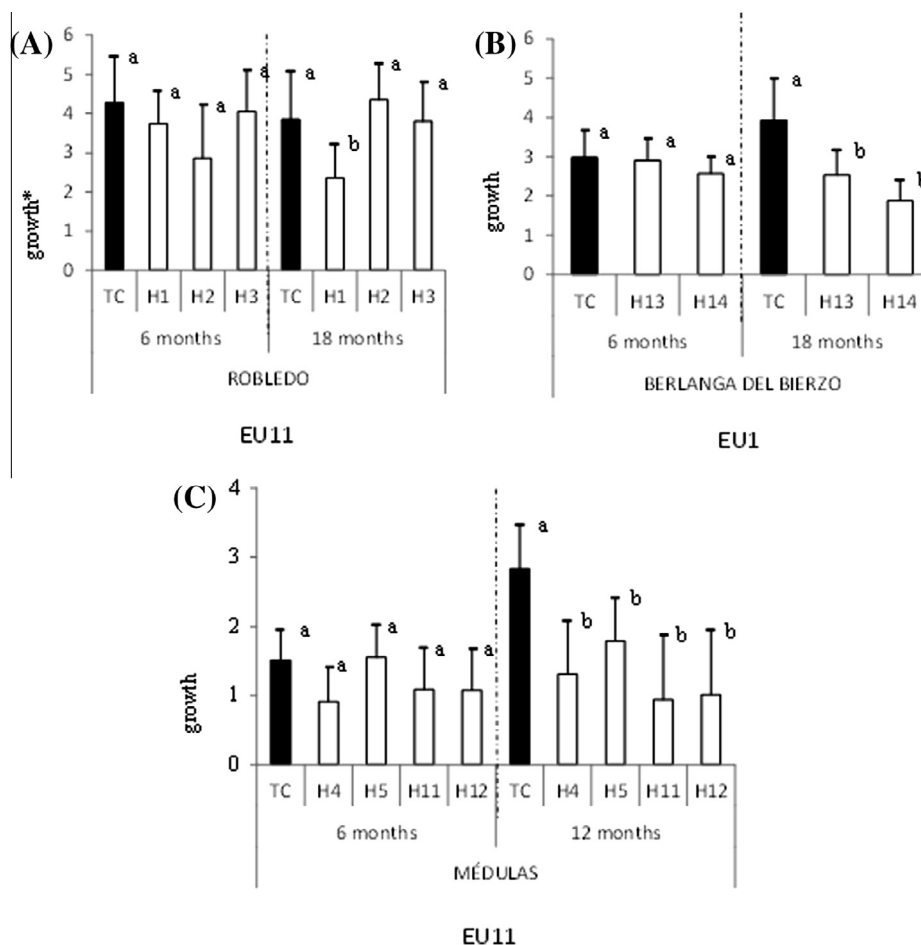


Fig. 5. Mean relative canker growth after treatment of inoculated (white bars) and non inoculated (black bars) cankers in Robledo (A), Berlanga del Bierzo (B) and Médulas (C) orchards after 6 and 12–18 months. Robledo and Médulas were inoculated with isolates of vc type EU11 and Berlanga del Bierzo was 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 $\ln(1 + \text{PIG})$, where $\text{PIG} = (A_j - A_i/A_i) * 100$ and A = area calculated with the ellipse formula.

very high but from 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.

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