Research Article

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Biological control of chestnut blight in Croatia: an interaction between host sweet chestnut, its pathogen *Cryphonectria parasitica* and the biocontrol agent *Cryphonectria hypovirus* 1

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Abstract

BACKGROUND: Chestnut blight, caused by the fungus *Cryphonectria parasitica*, is a severe chestnut disease that can be controlled with naturally occurring hypoviruses in many areas of Europe. The aim of this research was to measure the effect of different *Cryphonectria hypovirus* 1 (CHV1) strains on the growth of the fungal host and select strains that could potentially be used for human-mediated biocontrol in forests and orchards, and to investigate whether and how chestnut–fungus–virus interactions affect the development and growth of the lesion area on cut stems.

RESULTS: Two Croatian CHV1 strains (CR23 and M56/1) were selected as potential biocontrol agents. The sequencing of CHV1/ORF-A showed that both of these virus strains belonged to the Italian subtype of CHV1. *In vitro* transfection of selected virus strains from hypovirulent to genetically diverse virus-free fungal isolates and subsequent inoculation of all virus/fungus combinations on stems of genetically diverse sweet chestnut trees revealed that Croatian virus strain CR23 had an equally hypovirulent effect on the host as the strong French strain CHV1-EP713, while M56/1 had a weaker effect. Furthermore, it was shown that in some cases the same hypovirus/fungus combinations induced various degrees of canker development on different chestnut genotypes.

CONCLUSION: Some CHV1 strains belonging to the Italian subtype have similar hypovirulent effects on *C. parasitica* to those belonging to the French subtype. Furthermore, chestnut susceptibility and recovery could be influenced by the response of chestnut trees to particular hypovirulent *C. parasitica* isolates, and virus–fungus–chestnut interactions could have significant implications for the success of chestnut blight biocontrol.

Keywords: sweet chestnut genotype; *Cryphonectria parasitica*; fungal growth; hypovirulence

1 INTRODUCTION

Chestnut blight is a disease caused by the fungus *Cryphonectria parasitica* Murrill Barr. This ascomycete fungus infects the bark and cambium of chestnut trees through wounds and induces bark cankers which can lead to dieback of the distal parts after girdling branches or the entire tree trunk.[1] The fungus is native to Asia, where it coevolved with its hosts, *Castanea crenata* Siebold et Zucc. and *C. mollissima* Blume, which do not express serious disease symptoms.[2]

C. parasitica was accidentally introduced from Asia into North America at the beginning of the twentieth century and almost entirely destroyed American chestnut populations (*C. dentata* Marshall Borkh.).[3] In Europe this pathogen was first recorded on sweet chestnut or European chestnut trees (*C. sativa* Mill.) in Italy in 1938, from where it rapidly spread to almost all main chestnut-growing areas, causing dieback of a large number of chestnut trees along the advancing disease front. Two decades after the first disease records, infected European chestnut stands started to recover from the disease, as indicated by the occurrence of superficial, non-lethal chestnut blight cankers. This spontaneous recovery was found to be the result of the emergence of a hyperparasitic fungal virus in populations of *C. parasitica*.[4,5] *Cryphonectria hypovirus* 1 (CHV1), which also originates from Asia,[6] infects the fungal host and significantly reduces its virulence towards sweet chestnut trees, a phenomenon called hypovirulence.[7] Infection by the virus also inhibits sexual reproduction of the fungus and reduces its pigmentation and sporulation, giving it a typical white appearance in culture.[8] The discovery of transmissible hypovirulence

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provided the basis for biological control of chestnut blight disease.[9,10]

A non-encapsulated cytoplasmic RNA virus CHV1 belongs to the family Hypoviridae. Several genetically distinct subtypes of CHV1 (subtypes I, F1, F2, D and E) have been identified in Europe.[11] The Italian (I) subtype is widespread in southern and south-eastern Europe, from south-eastern France, across Italy, Switzerland, Slovenia, Croatia, Bosnia and Herzegovina, Macedonia and Greece to Turkey.[12–15] Subtypes F1 and F2 have been found in France and Spain and recently in Turkey,[16,17] while closely related subtypes E and D have been found in Spain and Germany.[11] Subtypes of CHV1 differ in their virulence towards *C. parasitica*.[18] Strains belonging to subtypes F1 and F2 are considered to be more virulent and inhibit the growth and sporulation of the fungal host on chestnut bark more efficiently than strains that belong to the Italian subtype.[13] These differences among CHV1 subtypes may have important consequences for biological control of chestnut blight disease.

Hypoviruses lack an extracellular phase and can be transmitted from one fungal individual to another via hyphal anastomosis (horizontal transmission), which provides the basis for biological control. The spread of hypoviruses has been restricted by the diversity of vegetative compatibility (vc) types of the fungal pathogen.[19,20] While the virus is transmitted very easily between compatible fungal strains, transmission efficiency is usually reduced between different vc types.[21] Hypoviruses can also be transmitted vertically into asexual spores (conidia) produced by infected fungal strains. In contrast, CHV1 has not been detected in sexual spores (ascospores) of the fungus.[22]

C. parasitica has been destroying sweet chestnut trees in Croatia for decades. Grafted marrons, appreciated for the exceptional quality of their fruits, appear to be very susceptible.[23] Chestnut blight was first reported in Croatia in 1955 in the western coastal region of Croatia, in Lovran County.[24] After its appearance, the disease spread through other sweet chestnut stands, causing significant damage to chestnut trees. In 1978, superficial cankers were first noticed in continental Croatian chestnut stands, and a large number of chestnut trees started to recover from the disease.[25] Thirty years later, hypovirulent isolates have been detected in each *C. parasitica* population studied, but prevalence of CHV1 significantly varied from as low as 12% (in coastal populations) to over 60% (in some continental populations).[26] Furthermore, high genetic diversity, which might obstruct natural dissemination of the CHV1 in populations of *C. parasitica* in Croatia, has been revealed using vc typing and SCAR markers.[26,27] Therefore, human-mediated biocontrol using well-defined CHV1 strains might be needed to complement naturally occurring hypovirulence in chestnut populations with low prevalence of hypovirulent *C. parasitica* strains, especially in marron orchards. For successful biological control, it is important to select appropriate CHV1 strains with an optimum effect on the fungal host and a high probability of spreading through chestnut populations.[15] Selected CHV1 strains could have different influences on distinct genotypes of the fungus.[28] Furthermore, to the best of our knowledge, the response of different genotypes of European chestnut to certain fungus/virus combinations has not been studied so far.

The main objectives of this study were: (i) to analyse the growth of hypovirulent *C. parasitica* isolates from Croatia and select CHV1 strains with strong and moderate effects on the original fungal host in which they were found in nature for further investigation of their effects in inoculation trials in cut chestnut stems; (ii) to transfect selected CHV1 strains to genetically diverse virus-free fungal isolates and determine the effect of each CHV1 strain on each fungal isolate; (iii) to determine the influence of various fungus/virus combinations on different naturally growing sweet chestnut genotypes.

2 MATERIALS AND METHODS

2.1 Hypovirulent *C. parasitica* isolates

A total of 28 CHV1-infected *C. parasitica* isolates used in this study were sampled from nine sweet chestnut populations throughout chestnut-growing coastal and continental areas of Croatia (Požega, PZ; Samobor, Sa; Cres, CR; Istria, IS; Ozalj, OS; Markuševac, M; Šamarica, ŠA; SIjeme, SLJ, and Hrvatska Kostajnica, HK). Based on RFLP analysis, all CHV1 strains found in *C. parasitica* sampled in Croatia were assigned to the Italian subtype.[26,29] All virus-infected *C. parasitica* isolates were kept in 22% glycerol at −80 °C and were recultured on potato dextrose agar (PDA) (Difco, Detroit, MI) for this research.

2.2 Fungal growth on PDA

The growth of 28 previously mentioned hypovirulent *C. parasitica* isolates was assessed on PDA. The experiment was performed according to Hillman *et al.*[30] Sterile petri dishes (9 cm in diameter) containing PDA (25 mL) were inoculated in the centre of the plate with mycelial plugs (4 mm in diameter) taken from the edge of fresh fungal cultures of isolates of *C. parasitica*. Plates were wrapped with parafilm and incubated at 24–26 °C under fluorescent light of approximately 2500 lux for a 16 h light and 8 h dark photoperiod. Following a lag phase of 24 h, the radial growth of each culture was determined by measuring twice the diameters of each culture at an angle of 90° after 3 and 5 days, in three replicate plates per isolate. Values of fungal growth after 3 and 5 days were presented as mean values ± standard deviation.

2.3 DsRNA extraction and RT-PCR

Two hypovirulent isolates expressing different growing properties on PDA were selected for further analyses. The isolate with the strongest effect on growth of its host was chosen – CR23, while one with a moderate effect on fungal growth was chosen randomly – M56/1. DsRNA was isolated as previously described by Allemann *et al.*[31] First-strand cDNA was synthesised from 100 ng of dsRNA using random primers (Promega, Madison, WI). PCR was performed using the primer pair for amplification of ORF-A, EP713-5/R2280, as described by Allemann *et al.*[31] After electrophoresis, PCR products from each sample were purified with the MiniElute PCR Purification kit (Qiagen GmbH, Hilden, Germany), and the concentration of the purified PCR products was measured spectrometrically.

2.4 Partial sequencing of selected CHV1 strains

A portion of the ORF-A region of each chosen CHV1 strain was amplified using primer combinations ep721-5 (CCGATTCCTTCAGTTGGTGC) and ep721-4 (GGAAGTCGGACATGCCCTG), modified after Allemann *et al.*,[31] and hvep1 (TGACACGGAAGCTGAGTGTC) and hvep2 (AGCGCGAATTTCTTGTCG), described by Gobbin *et al.*[32] Sequencing was performed using an ABI Prism 3130 Genetic Analyser (Life Technologies, Carlsbad, CA). Final nucleotide sequences of ORF-A were assembled using DNA Dynamo sequence analysis software and deposited in GenBank under the accession numbers KU904808–KU904809. The assembled sequences were compared between each other and with sequences of EP713 and Euro7 CHV1 available from the National Centre for Biotechnology Information (NCBI, Bethesda, MD).

2.5 Transfection of hypoviruses

We used four hypovirus-free *C. parasitica* isolates (L14, L44, L45 and L76) as recipients for CHV1 strains. The recipient hypovirus-free *C. parasitica* isolates were previously sampled in the Lovran area, in coastal Croatia, where naturally growing chestnut and grafted marron trees grow in mixed forest/orchards.[23] Each of these four *C. parasitica* isolates belongs to one of the four most dominant vc types, namely EU1, EU12, EU2 and EU17.[23] In each of them, three different CHV1 strains were transfected: CHV1-EP713, a strain that causes severe reduction in virulence in *C. parasitica*[13] (kindly provided by Dr Daniel Rigling), and two out of 28 hypovirulent isolates collected in Croatia: CR23, with a strong effect on the growth of the *C. parasitica* isolate in which it was originally found, and M56/1, with a moderate effect on the growth of its original fungal host. The effects of these viruses on the growth of the original fungi in which they were found were used as an indication of their potential effect on *C. parasitica* in general. Selected hypoviruses were transfected into virus-free fungal isolates by growing them next to the virus-infected isolates on PDA, resulting in 12 hypovirus/fungus combinations.[21] Virus transfection was considered to have been successful if the recipient fungus showed a change from orange to white phenotype, characteristic for *C. parasitica* isolates infected with CHV1. Each culture was checked for the presence or absence of hypovirus by extraction of dsRNA. To designate fungal isolates infected with a specific hypovirus, we added the hypovirus strain name in brackets to the *C. parasitica* isolate name, e.g. L14 (CR23). Four virus-free fungal isolates as well as all 12 virus-infected fungal isolates were tested for fungal growth on PDA, as described in Section 2.2 above.[30]

2.6 Genotyping of European chestnut individuals

Leaf samples from three randomly chosen European chestnut trees were collected from the surroundings of Samobor and designated as SG10, SG11 and SG18. DNA was extracted from fresh leaf tissue using the DNeasy Plant Mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. The DNA concentration was determined with NanoDrop (Thermo Fisher Scientific, Waltham, MA), and PCR reactions were carried out with 10 ng of template DNA. The PCR reaction contained: 1× PCR buffer, 1.5 mM of $MgCl_2$, 200 μM of dNTPs (Promega), 5 μM of forward and reverse primers and 0.5 U of GoTaq Hot Start (Promega) polymerase. Eight pairs of primers were used for the amplification of microsatellite loci: CsCAT1, CsCAT2, CsCAT3, CsCAT4, CsCAT6, CsCAT14, CsCAT17[33] and OAL[34] with fluorescently labelled forward primers. After the initial denaturation at 94 °C for 2 min, 35 three-step amplification cycles were performed: denaturation (94 °C, 30 s), annealing (45 s using different annealing temperatures for each primer pair, as described by Marinoni *et al.*[33] and Gobbin *et al.*),[34] and elongation (72 °C, 90 s), followed by a final elongation step (72 °C, 8 min). PCR products were separated by agarose electrophoresis to confirm successful amplification, and amplicons were sent to the Genescan Service of Macrogen (Seoul, Korea). Obtained chromatograms were analysed for allele sizes using GeneMapper 4.0 software (Applied Biosystems, Foster City, CA).

2.7 Fungal growth on chestnut stems

In addition, growth of CHV1-infected (hypovirulent) and control (virulent) fungal strains was also measured on stems of genotyped sweet chestnut trees. The stems were collected from a chestnut forest near Samobor in December 2013. Forty-eight chestnut stems (2–3 cm diameter) were harvested from three different, randomly selected trees (16 stems per chestnut genotype) and cut to approximately 50 cm length. Inoculation on stems was performed according to the procedure described by Fulbright *et al.*[35] and Lee *et al.*[36] The ends of the stems were sealed with Parafilm, and three small holes (2 mm diameter) per stem were made with a cork borer equidistantly at 15 cm on the stems to the depth of the cambium. Mycelial plugs from freshly grown *C. parasitica* cultures were inserted towards the cambium with a sterile scalpel, and the holes were sealed with Parafilm to prevent desiccation. Stems were kept in the dark at room temperature (20–22 °C) with high (~95%) humidity. Each *C. parasitica* genotype/virus combination (12 of them) and each *C. parasitica* control virus-free strain (four recipient fungal isolates) were inoculated 3 times on each of the three chestnut genotypes. Inoculations were made randomly on 16 stems of each chestnut genotype (three inoculations per stem). After 4 weeks of incubation, the major and minor semi-axes of each lesion were measured and their area calculated.

2.8 Data analysis

Statistical analyses of fungal growth on PDA and chestnut stems were performed using one-way analysis of variance (ANOVA) followed by a post hoc LSD test with Statistica 12 software (StatSoft Inc., Tulsa, OK).

3 RESULTS

3.1 Effect of different hypovirus isolates on *C. parasitica*

The majority of 28 hypovirulent isolates grew similarly, but few of them grew significantly slower than the others (Figs 1a and b). The fungal isolate CR23 stood out from all the others, with the slowest growth measured on the third and fifth days after inoculation (Figs 1a and b). Therefore, we used this isolate as a donor for transmission of its hypovirus into four virulent recipient isolates of *C. parasitica* in order to establish whether this virus had a strong effect on the growth of *C. parasitica* in general. In addition, isolate M56/1 was randomly selected for virus transmission among isolates harbouring viruses with a possible weaker impact on *C. parasitica*.

The *C. parasitica* isolates from Lovran in which CHV1-EP713, CR23 and M56/1 were transfected showed no, or little, reduction in growth on PDA plates (results not shown). However, transfected hypoviruses CHV1-EP713, CR23 and M56/1 had a significant effect on *C. parasitica* isolates L14, L44, L45 and L76 when tested on chestnut stems. Following inoculation on chestnut stems, significant differences were observed in lesion areas between isolates of *C. parasitica* infected with hypovirus strains and virus-free isolates (Fig. 2). The lesion area on chestnut stems caused by all virulent isolates was larger when compared with the lesion area caused by the same *C. parasitica* isolates infected with hypoviruses, except for isolate L44 on one of the used sweet chestnut genotypes.

3.2 Sequences of strong and moderate Croatian CHV1 strains

DsRNA isolation confirmed that virus strains CR23 and M56/1 were present in recultured *C. parasitica* isolates. A part of the genomic ORF-A was sequenced for both virus strains, CR23 and M56/1. The length of the analysed sequences was 1252 bp. Sequences of virus strains CR23 and M56/1 differed in 20 nucleotides and had 98% similarity between each other. Additionally, sequence similarity of virus strains CR23 and M56/1 was 98% each when compared with Euro7 strain of subtype I. However, the similarity was only 89 and

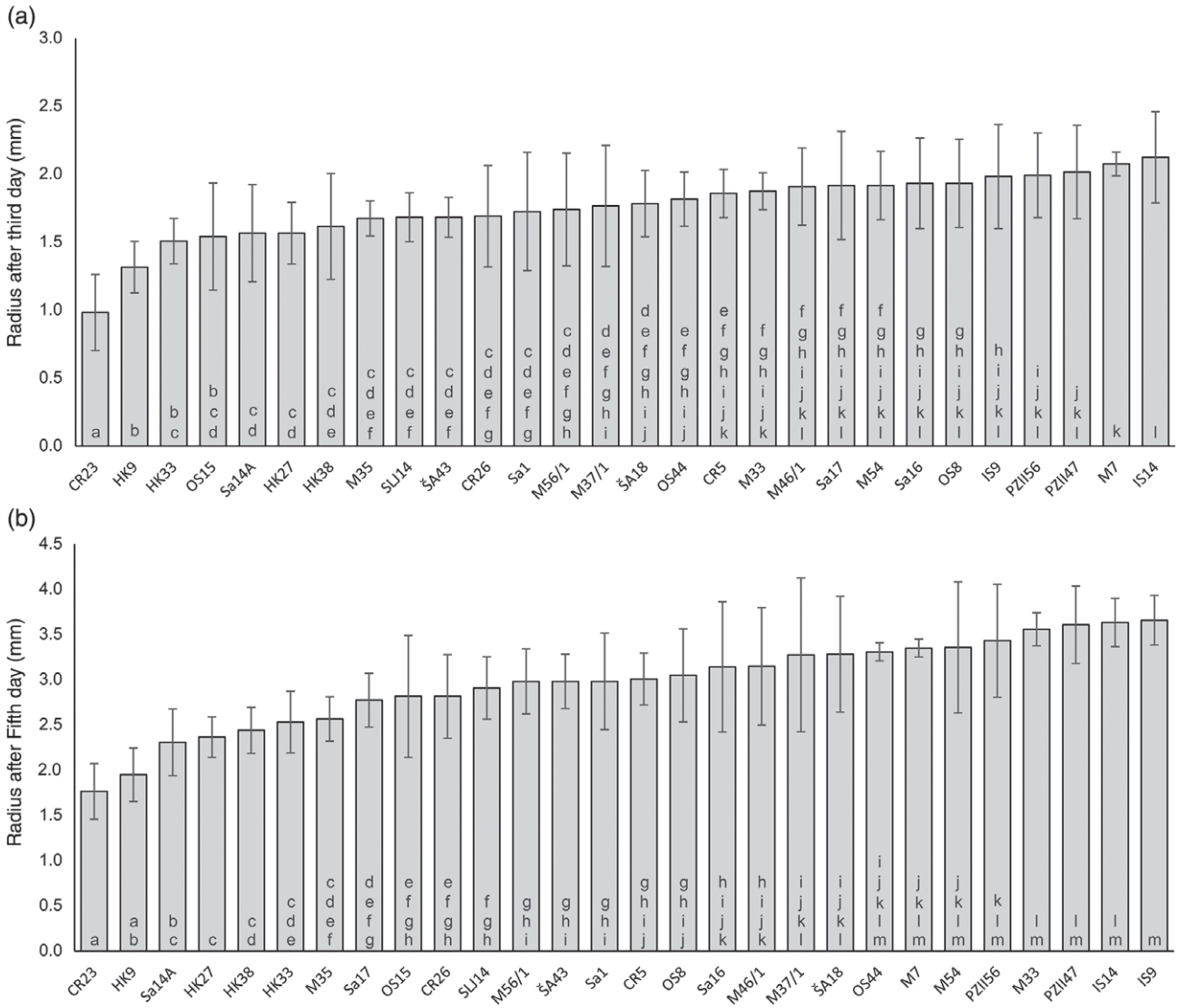

Figure 1. Growth of hypovirulent *C. parasitica* isolates from Croatian chestnut populations on potato dextrose agar medium 3 days (a) and 5 days (b) after inoculation.

88%, respectively, when compared with virus strain CHV1-EP713 of subtype F1. Therefore, it was confirmed that Croatian virus strains CR23 and M56/1 belong to the Italian subtype of CHV1.

3.3 Hypovirus–*C. parasitica*–sweet chestnut interactions

The genotyping showed that the three chestnut trees from which stems were derived for inoculation of fungal isolates represented different sweet chestnut genotypes, designated as SG18, SG11 and SG10. All eight analysed microsatellite loci were polymorphic (Table 1).

The lesion area on stems of all chestnut genotypes caused by genetically different virulent fungal isolates, namely L14, L44, L45 and L76, that belong to different vc types, EU1, EU12, EU2 and EU17, respectively, is shown in Fig. 2. Results revealed that sweet chestnut genotypes SG11 and SG10 were most severely affected by fungal isolate L76, which produced larger lesions on stems of these genotypes, compared with the other three virulent fungal isolates, while chestnut genotype SG18 was equally affected by all fungal isolates. Comparison of lesions caused by the same virulent fungal isolate on different chestnut genotypes showed that fungal isolates L14, L44 and L45 produced the largest lesions on stems of chestnut genotype SG18, while isolate L76 produced the largest lesions on stems of chestnut genotype SG10. An especially small lesion area was caused by virulent isolate L44 on stems of chestnut genotype SG10.

Although virus transmission to virulent *C. parasitica* isolates generally resulted in a decreased lesion area, differences in the effect of all transmitted hypovirus strains on the genetically diverse fungal hosts were observed (Fig. 2). The effect of each viral strain on each fungal isolate was not the same, and the final outcome was influenced by sweet chestnut genotype and specific virus/fungus combination (Fig. 2). In extreme cases, transmission of virus strains CHV1-EP713 and M56/1 to fungal isolate L44 did not result in significant changes in lesion area on stems of chestnut genotype SG10, while a significant decrease in lesion area was measured on stems of other chestnut genotypes. Furthermore, virus/fungus combination L45 (EP713) produced larger lesions on stems of chestnut genotype SG10 than on stems of chestnut genotype SG11, and L76 (EP713) produced larger lesions on stems of chestnut genotype SG18 than on stems of chestnut genotype SG11.

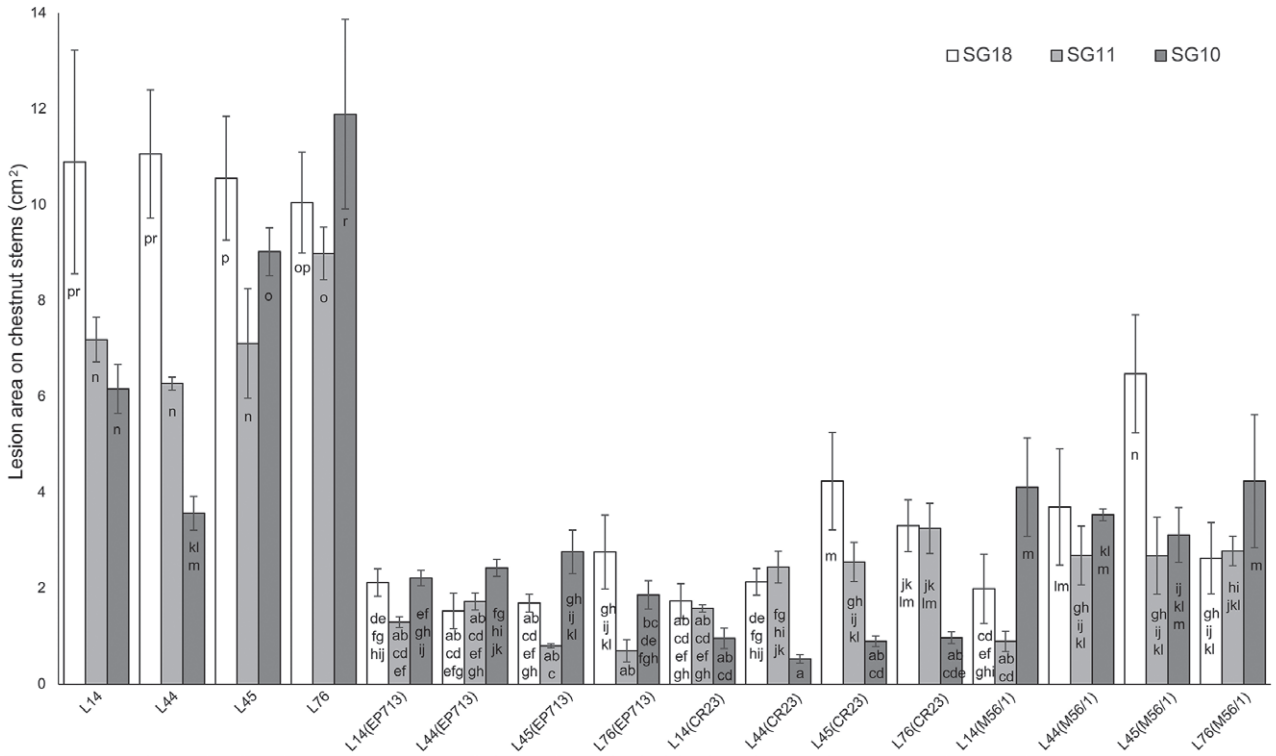

Figure 2. Growth of four virulent and 12 hypovirulent *C. parasitica* isolates on chestnut stems after 4 weeks of incubation. Different shades (white, grey and dark grey) of bars represent three different genotypes of chestnut.

Table 1. Genotypes of sweet chestnut trees

Chestnut genotype	CsCAT1		CsCAT3		CsCAT4		CsCAT6		CsCAT17		OAL		CsCAT14		CsCAT2	
SG18	216	222	227	229	220	236	194	194	138	142	299	299	160	160	216	216
SG11	193	208	229	243	213	213	172	177	138	156	299	331	141	160	206	212
SG10	216	216	197	225	220	238	172	180	142	146	299	299	133	160	206	206

Also, virus/fungus combinations L14 (M56/1) and L76 (M56/1) produced larger lesions on stems of chestnut genotype SG10 than on other chestnut genotypes, and L45 (M56/1) produced larger lesions on stems of chestnut genotype SG18 than on other chestnut genotypes. Virus strain CR23 had a variable effect on fungal isolates L44, L45 and L76, most strongly reducing growth of these isolates on stems of chestnut genotype SG10 when compared with the other chestnut genotypes.

Comparison of French virus strain CHV1-EP713 and Croatian strain CR23 showed that the former had a stronger effect only on fungal isolate L45 when this isolate was inoculated on stems of genotypes SG18 and SG11, and on isolate L76 when inoculated on stems of sweet chestnut genotype SG11. The Croatian strain CR23 had a stronger effect than strain CHV1-EP713 on fungal isolates L14, L44 and L45 when these fungal isolates were inoculated on chestnut genotype SG10. However, in the majority of virus/*C. parasitica*/sweet chestnut combinations, the effects of these two virus strains belonging to different CHV1 subtypes were similar; there was no considerable difference in lesion size. Considering all these results together, the French strain CHV1-EP713 and the Croatian strain CR23 can be considered to be virus strains with equally strong hypovirulent effects on the host. The Croatian virus strain M56/1 was proven to be weaker than CR23 and CHV1/EP713. Although virus strain M56/1 had a similar effect to viruses CR23 and CHV1-EP713 on fungal isolate L14 when this isolate was inoculated on stems of genotypes SG18 and SG11 and on isolates L44 and L76 when these isolates were inoculated on stems of genotypes SG11 and SG18, respectively, in all other virus/*C. parasitica*/sweet chestnut combinations the isolate M56/1 had less effect on reduction in lesion area than CR23 and/or CHV1-EP713.

4 DISCUSSION

Our aim was to select CHV1 strains that would be appropriate for human-mediated biocontrol in orchards and/or sweet chestnut forests, as well as to evaluate how virus–fungus–chestnut interactions could influence success or failure of natural and human-mediated biocontrol. The hypovirus strains selected for further testing, CR23 and M56/1, showed strong and moderate effects on the original fungal hosts in which they were found in nature, respectively. Testing of their applicability for biological control was performed by analysing the effect of these viruses on genetically diverse virus-free fungal isolates into which they were transferred *in vitro*. Although the growth of fungal isolates on PDA after virus transfection did not always show the expected decrease, the initial assumption that CR23 had a stronger hypovirulent effect

than M56/1 was confirmed in trial inoculations on chestnut stems. In most of the virus–fungus–chestnut combinations, strain CR23 had a stronger effect than strain M56/1, and only in a few cases were the effects of strains CR23 and M56/1 not significantly different. Furthermore, in the case of L44, which, although virus free, grew very poorly on chestnut genotype SG10 (probably owing to fungus–chestnut interactions), the transfection of the fungus with EP713 and M56/1 did not cause any significant difference in the induced lesion area on the same chestnut genotype, while transfection with CHV1 strain CR23 caused the formation of significantly smaller lesions. This implies that only particular virus strains or virus/fungus combinations might be effective as biocontrol agents on certain chestnut genotypes.

Analysis of mycelium growth on PDA showed that virus-free isolates grew faster than virus-infected isolates, although under certain conditions the growth of the fungal isolates infected with CHV1 belonging to subtype I was faster than the growth of uninfected isolates on PDA.[18,37] Furthermore, in the survey of Sotirovski *et al.*,[37] mycelial growth *in vitro* was not highly correlated with canker growth or callus formation in the field; therefore, it was not considered to be a good predictor of hypovirulence-inducing potential. In our case, the growth of different combinations of fungus/virus isolates was tested on PDA and on sweet chestnut stems, revealing the need to include the genotype factor of chestnuts when predicting the possible effect of fungus/virus combinations in biological control in the field. The test on PDA might give some indication of which virus strain could have a potentially strong hypovirulent effect, but this is far from reliable.

Results concerning canker growth on sweet chestnut stems revealed that strain CR23, which belongs to Italian subtype I, not only is the Croatian virus strain with the strongest hypovirulence effect but also had a similar effect on *C. parasitica* to strain CHV1-EP713, which belongs to French subtype F1. CHV1 subtypes were previously shown to have a different impact on *C. parasitica*.[18] French subtypes of CHV1 are thought to inhibit the growth of *C. parasitica* on living chestnut bark more strongly than virus isolates belonging to the Italian subtype.[13] However, Bauman[28] showed that there was no difference in the size of cankers produced by the same fungal isolates infected with strain CHV1-EP713 and Euro7. Our study confirms that the effect of Croatian virus strain CR23 (which belongs to the Italian subtype) on the host is as strong as the effect of strain CHV1-EP713, a strain that belongs to the French subtype with a proven strong effect on *C. parasitica*.[7,13] CHV1 subtype I is known to have a better ecological fitness compared with the French subtypes F1 and F2.[13,18] Furthermore, according to previous attempts at biocontrol of chestnut blight, viruses that belong to subtype I might be more appropriate biological control agents than French subtypes F1 and F2 and could have a long-term biocontrol effect due to the higher probability of becoming established in fungal populations in which they are introduced. In several European countries, hypovirulent strains have been applied for biocontrol of chestnut blight.[1,2,13,38–40] Hypovirulent isolates of *C. parasitica* previously naturally spread in those regions or in geographically close regions were usually used in such attempts. After an experimental release of hypovirulent strains, CHV1 subtype I quickly established and spread in populations of *C. parasitica* within sweet chestnut populations. On the other hand, after the introduction of *C. parasitica* strains infected with CHV1 of the subtype F1, viruses belonging to this subtype neither persisted nor became established in sweet chestnut coppices or orchards, although they may still be applicable for enabling quick healing of individually treated cankers in orchards and plantations.[13] In the United States, both French and Italian subtypes of CHV1 from Europe have been used for biocontrol, because of inadequate naturally occurring hypovirulence. Attempts to treat American chestnut (*Castanea dentata*), which is highly susceptible to chestnut blight, have mainly been unsuccessful, and CHV1 could not be reisolated later from treated chestnut sites.[41,42] However, it seems that recent release of the weaker Euro7 hypovirus strain, which belongs to the Italian subtype, has resulted in spreading of the virus and induction of healing cankers on the diseased trees.[43]

An attempt at biological control was conducted in Croatia 30 years ago using *C. parasitica* isolates infected with CHV1 of the French subtype.[25] Five years after treatment, the recovery of some sweet chestnut trees was observed. However, in our recent research, CHV1 strains belonging to the French subtype have not been found anywhere in Croatia.[26] Therefore, the French subtype might have been successful for a short time after its release, but there is no evidence of persistence of this CHV1 subtype in Croatian *C. parasitica* populations. Accordingly, CHV1 subtype I, which is present to varying extents in Croatian *C. parasitica* populations, would be the most logical choice for biological control of chestnut blight in Croatia. The strong effect of the virus strain CR23 on the fungal host, comparable with that of strain CHV1-EP713, may have a positive effect on the efficiency of this virus as biocontrol agent; conversely, it may also affect its fitness and capability to spread within the fungal population, in which case 'weaker' isolates, such as M56/1, may be better suited as a biological control agent. Therefore CR23 could be used for direct inoculations of sweet chestnut trees in orchards, whereas M56/1 could be used in forests, because its natural spread after inoculation could be expected.

Previous studies analysed interactions between genetically different isolates of *C. parasitica* fungus and various hypoviruses, indicating that each hypovirus interacts with the host differently and that the fungal genome can play an important role in hypovirus dissemination.[28] This is confirmed by the results of our study, where four different genotypes of the fungus were used. A third component of this pathosystem was also included in our study – the sweet chestnut. In general, virulent isolates produced larger lesion areas on chestnut stems than hypovirulent isolates, which is consistent with previous studies.[18] However, it was shown that fungal isolates differed substantially in their virulence towards the sweet chestnut genotypes tested. Conversely, chestnut genotypes were not equally susceptible to tested fungal isolates. It was previously suggested that CHV1 infection may create hypovirulent fungal strains that are not uniform in all biological control characteristics.[28] This study further showed that some virus/fungus combinations do not have an equal impact on different sweet chestnut genotypes, pointing to the important role of chestnut genotype in disease development and biological control. The response of different genotypes of chestnut could be as important as the characteristics of the fungus (fungi) and virus/es to the overall outcome of the disease, and this could have significant implications for the success of human-mediated biological control. Weaker recovery of 'Lovran Marron', an indigenous Croatian chestnut cultivar, in comparison with naturally growing trees in the presence of well-established natural hypovirulence was reported before.[23] Those results already showed that this chestnut genotype is especially vulnerable to chestnut blight, as this particular cultivar did not recover well from the disease, even in the presence of hypovirulent *C. parasitica* isolates in cankers. This research reveals that there is a difference in susceptibility of naturally growing trees to different virulent and hypovirulent *C. parasitica* isolates.

Hypovirulence remains the best way for successful biological control of chestnut blight. However, predicting the success or failure of hypovirulence for biological control is not simple, as three interacting factors, the chestnut blight fungus, the hypoviruses as biocontrol agents and the chestnut, are involved. In this study, differential response of particular sweet chestnut genotypes to infection with various virulent and hypovirulent *C. parasitica* isolates was observed. Therefore, forest recovery could be influenced not only by the spread of CHV1 but also by the response of chestnut trees to particular hypovirulent *C. parasitica* isolates. Considering the complexity of interactions among all three components, it may be extremely difficult to find one hypovirus that would produce the optimum effect in all virus–fungus–chestnut combinations, especially in situations where diversity of fungal populations is very high, as reported for Croatia,[23,26] and especially when applying biocontrol through hypovirulence in forests where diversity of chestnuts is higher than in orchards.[23,44] Simultaneous application of more than one hypovirus (strong and moderate) might be a possible strategy that would yield the optimum biological control outcome in such conditions.

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