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ORIGINAL ARTICLE

New formulation and delivery method of *Cryphonectria parasitica* for biological control of chestnut blight

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bioavailability, carrier, *Castanea sativa*, HPMC, hypovirulence.

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Abstract

Aims: This study aimed to develop a new formulation of *Cryphonectria parasitica* hypovirulent mycelium suitable for inoculations of tall trees from the ground. *Cryphonectria parasitica* hypovirulent strains are widely used for biological control of chestnut blight. However, it is often inconsistent and ineffective not only for biological reasons but also because the current manual application of hypovirulent strains on adult plants is difficult, time-consuming and expensive. Here, we propose an improved formulation and more effective mode of application of hypovirulent strains, which could boost chestnut blight biocontrol.
Methods and Results: The Cp 4.2H hypovirulent strain was formulated as mycelium discs with polyethylene glycol and hydroxypropyl methylcellulose, loaded into lead-free pellets that are used as carriers to inoculate cankers on chestnut stems by shooting. The formulation of mycelium did not hamper its viability which was stable, with an estimated shelf life of 72 days at 6 ± 1°C. The inoculum effectiveness was confirmed *ex planta* and *in planta* in a small-scale pilot study in field, where formulated mycelium discs of hypovirulent strain Cp 4.2H were inoculated by airgun shot method into the chestnut bark. *In planta*, Cp 4.2H was recovered in 37% of bark samples taken around the inoculated points 1 year after the treatment.
Conclusions: We demonstrated that the proposed airgun shooting inoculation method of *C. parasitica* hypovirulent strain formulated as mycelium discs is suitable for treatment of adult chestnut trees.
Significance and Impact of the Study: The proposed method could be a valid alternative to the traditional manual technique of chestnut biocontrol. The main advantages are the cost-effectiveness and the ease to treat high-positioned, otherwise unreachable cankers both in orchards and forests.

Introduction

Cryphonectria parasitica (Murrill.) M.E. Barr, is the necrotrophic ascomycete responsible for chestnut blight, a detrimental disease of chestnuts in North America and Europe (Gryzenhout *et al.* 2009). *Cryphonectria parasitica* causes cankers, plant dieback or death. The overall damage ranges from loss of timber and nuts to modification of the ecosystem due to the substitution of chestnuts with other species (Loo 2009). Recently, the disease has emerged in new countries in Europe (https://gd.eppo.int/taxon/ENDOPA/distribution, accessed July 2016), and the restoration of infected chestnut forests is far from being accomplished.

In the 1950s, Biraghi (1953) described healing cankers on the European chestnuts, *Castanea sativa* and subsequently, the other researches proved the existence of hypovirulent strains in these cankers (Heiniger and Rigling 1994). Hypovirulence is related to *Cryphonectria hypovirus* (CHV), the species of dsRNA viruses which parasitize the fungus (Choi and Nuss 1992) and reduce its virulence. The transmission of the hypovirus to the

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pathogen occurs via anastomosis or asexual spores. Since this discovery, the scientific community recognized hypovirulence as a potential tool for disease management; however, there are still difficulties in obtaining effective chestnut blight control.

Several papers highlighted the most critical issues in chestnut blight biocontrol (Heiniger and Rigling 1994; Peever *et al.* 2000; Milgroom and Cortesi 2004; Mcdonald and Double 2005; Robin *et al.* 2010). In *C. parasitica,* a self/nonself recognition system genetically regulates the horizontal transmission of cytoplasmic material as well as viruses (Cortesi and Milgroom 1998; Cortesi *et al.* 2001). Variability in the self/nonself recognition system in the fungal populations is one of the major factors influencing the diffusion of hypovirulence. The virulence of CHV types (Chen and Nuss 1999; Peever *et al.* 2000; Bryner and Rigling 2012), their fitness and diverse epidemiological factors are other reasons partially explaining the experimental transmission rates divergent from the theoretical ones (Zhang *et al.* 1998; Peever *et al.* 2000; Dawe and Nuss 2001; Milgroom and Cortesi 2004; Nuss 2005; Robin *et al.* 2010).

Moreover, to successfully manage chestnut blight, we have to consider not only biological aspects underlying hypovirulence, but also those regarding specifically the treatment, that is, the release of hypovirulent strains. Components of hypovirulent mycelium formulations and delivery methods are often underestimated issues; however, they can deeply contribute to the success of biopesticides (Whipps and McQuilken 2009). The stability of the inoculum, the widespread distribution and prompt bioavailability are all fundamental requirements for an effective biocontrol. To our knowledge, in chestnut blight management, no studies have critically evaluated these aspects so far. The deployment of *C. parasitica* hypovirulent strains is critical, in particular for older trees. After the early attempts (Grente 1981; Griffin 1983; Garrod 1985; Turchetti and Maresi 1988), no more attention has been given to improve inoculation methods. Meanwhile, biological control has received increasing attention, and this has led to the introduction of more efficient microbial formulations and delivery methods on the market (Fravel 2005).

The chestnut recovery programmes adopted different types of inocula and formulations, ranging from mycelium to conidia, but the mycelium formulations were the most extensively employed (Heiniger and Rigling 1994). The standard inoculation method used to evaluate the hypovirulence establishment and to test selected resistant chestnuts in nature is still based on manual punching and removing the bark, and inserting agar plugs or slurry mycelium underneath the bark all around the canker. Although quite precise, this method has several drawbacks—it is time-consuming, requires qualified personnel and is applicable only to certain cankers on trees, in particular the young and easily accessible ones.

A commercial product based on *C. parasitica* hypovirulent strain should be applicable both in plantations and forests, where the decision to employ control procedures depends on ease of their employment and sustainability. A formulated product that is stable and easy to deliver can ultimately enhance the treatment efficacy and, at the same time, could help us to improve experimental designs and to draw stronger conclusions from biological control bioassays (Milgroom and Cortesi 2004).

In this study, we propose a new method of inoculation, which guarantees an adequate delivery of a *C. parasitica* hypovirulent strain and overcomes some of the drawbacks of biological control techniques applied so far, in particular, the impossibility to reach higher cankers by operators. We developed a formulation of *C. parasitica* suitable for deployment on tall trees from the ground and evaluated it for (i) persistence of the bioactive control agent during conservation, (ii) efficacy of hypovirulent strain delivery and (iii) its survival *in vivo* and in the natural context.

Materials and methods

Inoculum preparation and formulation

The strain used in this study, Cp 4.2, was randomly selected within the most frequent vc type EU-2 of a population of *C. parasitica* sampled in the 'Parco delle Colline', a sub-urban park in Brescia city, Italy. The park is 4300 ha big, and 30% of the area comprises chestnut stands. Cp 4.2 was converted to hypovirulence with E13 hypovirulent strain containing CHV1 virus, as described previously (Cortesi *et al.* 2001). Virulent Cp 4.2 and hypovirulent Cp 4.2H strains were grown in liquid culture adding 10 agar mycelium plugs, taken from the edge of a 14-day-old colony, into a flask containing 100 ml of potato dextrose broth (Oxoid, Hempshire, UK) supplemented with tetracycline (50 mg l^{-1}). The flasks were shaken at 100 rev min^{-1} with an orbital shaker (mod. M201-OR; MPM Instruments, Bernareggio, Italy) for 7 days at 25°C. Subsequently, the mycelium was vacuum-filtered through two layers of gauze and dried at 37°C for 48 h.

The dried mycelium was ground in the ultra centrifugal mill (mod. ZM200; Retsch, Hann, Germany) with 0·25 mm sieve, 12-teeth rotor rotating at 2012·4 ***g***. Grinding efficiency equal to percent ratio of mycelium recovered with respect to mycelium ground, disc preparation and overall yields were assessed. The powder was mixed in mortar with polyethylene glycol (3–4% w/w,

PEG 400; ACEF, Fiorenzuola D'Arda, Italy), hydroxypropyl methylcellulose (32–39%, HPMC; Methocel E5; The Dow Chemical Company, Midland, MI, USA) and water (39–50%) for 3 min to obtain a homogeneous semisolid and sticky paste, which was laminated to 1·5 mm thickness by a manual twin roll calendar, and dried at 25°C for 12 h. The dry films were die-cut into discs of 2·5 mm diameter.

Technical and biological properties were assessed both for hypovirulent and virulent mycelia on 10 randomly selected discs. The technical parameters were: average weight, thickness, diameter of the discs and biological active ingredient concentration (%). The biological parameters were: viability of the dried and ground mycelium (not formulated) and formulated mycelium, and fungal concentration in the formulated discs at t_0. Before formulation, 1 mg of mycelium was suspended in 1 ml of sterile water containing 0·02% Tween80 (Sigma-Aldrich) in an Eppendorf tube, and the tube was vortexed for 30 s. Serial dilutions were streaked on malt extract agar (Oxoid). The colony-forming units (CFU) were counted after 7 days of incubation and the viability estimated on three replicates was expressed as log (CFU) per mg of mycelium. The fungal concentration in the formulated discs at t_0 was estimated as follows: discs were soaked for 1 h in 1 ml 0·02% Tween80 in sterile water. CFU-counts were recorded following serial dilutions of the suspension, and expressed as previously described.

Erosion study

Erosion of the system was evaluated by placing three discs accurately weighted in a tissue bag (Shandon™ nylon biopsy bags small; Thermo Scientific™, Waltham, MA). The bags were placed in a tube of a disintegration test apparatus (DT3; Sotax, Thun, Switzerland) oscillating at 25 strokes per min in 800 ml of distilled water at room temperature ($N = 3$). The bags were removed after predetermined time periods, placed in oven at 60°C for 24 h and weighted. The erosion was calculated as per cent ratio between the final weight of the dry mass and the initial weight of the discs.

Shelf life of the mycelium discs

Mycelium discs were stored in refrigerator at 6 ± 1°C. Their shelf life was estimated every month for 6 months as fungal concentration of each disc, as described above. The time required for 50% decrease of the initial fungal concentration (DT50) was calculated on six replicates, using a first-order kinetic model.

Three randomly chosen colonies were used to verify the strain identity and presence of the CHV1 virus, which was determined based on white phenotype of the mycelium and the ability to convert the original virus-free isogenic strain to hypovirulence, as previously described. Virus transmission was confirmed by observing mycelium morphology change in the recipient strains (Cortesi *et al.* 2001). Mating type and fingerprinting analysis were also performed to check the identity of re-isolated strains (McGuire *et al.* 2004; Davis *et al.* 2005).

Mycelium discs delivery method

Each *C. parasitica* mycelium disc was glued with an aqueous solution of povidone (15% w/w, Kollidon 30; BASF, Ludwigshafen, Germany) within the concavity of a hollow point lead-free 4·5-mm diameter pellet (Crosman corp., Bloomfield, NY, USA). The pellet, loaded either with the virulent or the hypovirulent formulation, was shot from a 10 m distance in the chestnut bark with a Weihrauch HW977 airgun lever action rifle cal. 4·5 mm (Weihrauch & Weihrauch GmbH & Co. KG, Mellrichstadt, Germany), so that the pellet entered into the 1–1·5-cm depth within the bark.

To assess the delivery method, bioassays were conducted in controlled conditions and in field.

In controlled conditions, chestnut stems collected in winter, about 10 cm in diameter and 45 cm long, were shot with one pellet loaded either with the virulent or the hypovirulent strain. As control, agar mycelium plugs of each formulation were inserted within cork-borer bark wounds, as previously described (Peever *et al.* 2000). Each treatment was carried out in six replicates. After inoculation, the top of each chestnut stem was covered with a layer of paraffin, and the bottom was dipped in 5 cm water and incubated in a growth chamber at 24 ± 1°C, 85% RH and 12-h photoperiod. Four weeks after inoculation, the area of each canker was calculated by measuring the length (L) and the width (W) on the perpendicular axes of each canker and by applying the formula for an ellipse ($\pi LW/4$).

The delivery method was also assessed in the field, inoculating 20 chestnut stems at 2–5 m height, shooting pellets loaded with mycelium discs of Cp 4.2H hypovirulent strain only. Hand wound inoculations were carried out as previously described on the same number of randomly chosen stems ($N = 20$). In addition, one natural canker was treated shooting the pellets close to the margin around the canker. As control, stems were inoculated with pellets not loaded with the mycelium. One year after the inoculation, 16 randomly chosen bark wounds were measured and analysed for the presence of the Cp 4.2H released strain, bark samples were then taken around the point of entrance of the pellet to assess the presence of hypovirulent strain**.** In addition, in the same area, we

sampled an untreated canker (un-inoculated control) to isolate *C. parasitica* strains naturally occurring in the field. Isolation and purification were performed as previously described (Cortesi *et al.* 1996).

Strains isolated from field samples were characterized for CHV1 presence, vc type, mating type and fingerprinting, to ascertain if they were the inoculated and pellet-delivered strain. Presence of CHV1 virus was assessed as previously described. The vc type was determined by pairing the reisolated strains with those delivered and with the EU-2 tester, the most frequent vc type found in the area, as previously described (Cortesi *et al.* 2001). Mating type and fingerprint analysis was performed as previously described (McGuire *et al.* 2004; Davis *et al.* 2005) to confirm that the strain inoculated and recovered was the same.

Statistical analysis

Mycelium viability and canker area data were analysed by Student *t* test using R software, ver. R3·0·2. (R Core Team 2013).

To determine the half-life of the formulated strains at the storage conditions described, we fitted the CFU data to a first-order kinetic equation: $t = 1/k \times \ln(C_0/C_t)$ where k = rate constant, C_0 = initial concentration, C_t = concentration at time t. The calculated slope of the model k (rate of the first-order kinetic reaction) was then used to estimate the DT50. The fit was performed using Microsoft Excel software after log transformation of CFU.

Results

Mycelium formulation

Mycelium of hypovirulent and virulent strains grown in liquid culture was filtered, dried and finely ground. The powder was added with excipients for the preparation of discs. The yields after grinding and disc preparation steps ranged between 73% and 85% for Cp 4.2H and Cp 4.2, respectively, and the overall formulation yields were 58% and 72% for Cp 4.2H and Cp 4.2 respectively (Table 1). The mycelium concentration in the formulated discs was 30% (w/dry w) with a titre ranging between 3·8 log (CFU per mg) and 4·1 log (CFU per mg) (Table 2). The formulation did not affect significantly the fungus viability as confirmed from the Student *t* test for both strains: $P = 0{\cdot}1394$ and $P = 0{\cdot}5172$ respectively (Table 3). In order to verify the complete release of the strain following hydration, the weight loss of the discs was evaluated *in vitro* following immersion in water. The relatively high water solubility and low viscosity of the cellulosic polymer used for the formulation allowed complete disc erosion within 60 min (Fig. 1).

Table 1 Yields of formulated *Cryphonectria parasitica* mycelium

Strain	Yield (%)	
	Cp 4.2H*	Cp 4.2
Grinding efficacy	79·4	83·0
Disc preparation	73·3	85·7
Overall formulation	58·2	71·2

*CHV1 infected strain.

Table 2 Properties of formulated mycelium discs of *Cryphonectria parasitica*

Property*	Strain	
	Cp 4.2H†	Cp 4.2
Weight (mg)	5·73 ± 0·72	6·82 ± 0·79
Thickness (mm)	1·23 ± 0·29	1·22 ± 0·07
Diameter (mm)	2·45 ± 0·01	2·48 ± 0·07
Mycelium content (mg)	1·7	2·0
Viability (log CFU per mg) at t_0	3·8 ± 1·2	4·1 ± 1·36

*N = 10 discs.
†CHV1-infected strain.

Formulated mycelium shelf life

The number of viable propagules of Cp 4.2H discs after 6 months of storage at 6 ± 1°C declined from 3·8 to 2·1 log (CFU per mg) whereas for Cp 4.2, the reduction was lower, from 4·1 to 2·9 log (CFU per mg) (Table 4). The estimated DT50 for the two strains were 72 and 85 days respectively (Table 4; Fig. 2). After 6 months of storage, we re-isolated the mycelia from formulated discs to check for hypovirulence, the lack of contamination and the strain morphological and genotypic characters. We did not detect any contaminants in the formulated discs. The morphological characters, the presence of the CHV-1 hypovirus in Cp 4.2H, the mating type, *MAT*-1, and the fingerprint profile of the two strains before and after the formulation were identical (results not shown).

Delivery method

We assessed the bioavailability of the mycelium delivered by shotgun in two experiments, the first *ex vivo* in controlled conditions, the other in a small-scale field trial. In controlled conditions, all inoculations originated cankers, independently of the method used. However, by shotgun delivery, the areas of the cankers were respectively six- to eightfold bigger than those originated by the standard cork-borer wound inoculation, both for the hypovirulent Cp 4.2H ($P = 4{\cdot}199 \times 10^{-3}$) and for the virulent Cp 4.2

Table 3 Viability of *Cryphonectria parasitica* mycelium before and after formulation at t_0

Strain	Concentration (log CFU per mg)				
	Preformulation*	Postformulation†	*T* value	*P* value	CI
Cp 4.2H	1·686	1·188	2·3893	0·1394	−0·398; 1·394
Cp 4.2	1·775	1·674	0·7797	0·5172	−0·459; 0·662

*Preformulation refers to mycelium dried and ground.
†Postformulation refers to mycelium formulated as discs.

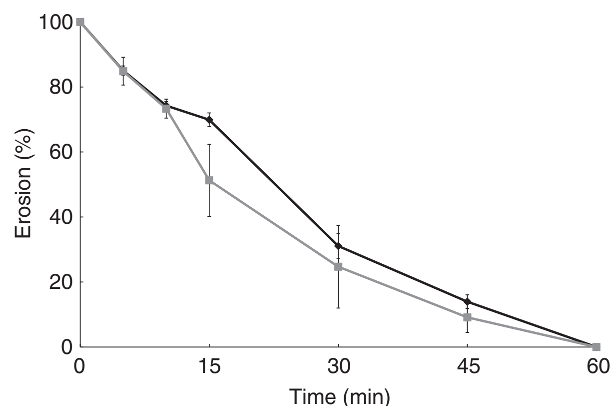

Figure 1 Water erosion profiles of *Cryphonectria parasitica* mycelium formulated discs. The Cp 4.2 strain is depicted in grey, the Cp 4.2H strain in black. Bars indicate standard errors.

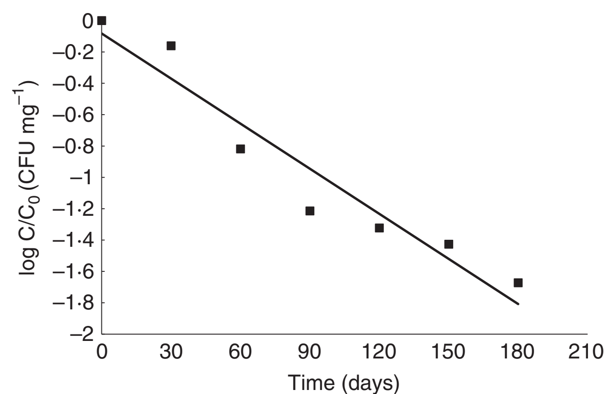

Figure 2 First-order kinetic model for shelf life of *Cryphonectria parasitica* hypovirulent formulated mycelium Cp 4.2H.

Table 4 Model parameters and DT50 estimated values for discs of *Cryphonectria parasitica* formulated mycelium

Strain	CFU per mg at		Kinetics		
	t_0	endpoint	*k*	R^2	DT50 (days)
Cp 4.2H	3·8	2·1	−0·0096	0·9263	72·2
Cp 4.2	4·1	2·9	−0·0081	0·5915	85·6

formulations ($P = 9{\cdot}182 \times 10^{-9}$; Fig. 3). In the field, where we delivered only Cp 4.2H in randomly chosen trees, the cankers developed following shotgun inoculations were on average $4{\cdot}83 \pm 0{\cdot}29$ cm^2, not significantly different from those obtained by hand wound inoculation, which averaged $3{\cdot}61 \pm 1{\cdot}46$ cm^2. We isolated 20 *C. parasitica* strains from 10 out of 16 bark samples taken from the shotgun cankers. All purified strains were analysed for the mating type, vegetative compatibility group and fingerprint profile. We identified 11 strains from six samples as Cp 4.2H (Table 5), and the conversion test confirmed the presence of the CHV-1 hypovirus in all of them. In three samples, 6, 11 and 16, we found Cp 4.2H in association with other *C. parasitica* strains, characterized by opposite mating type and with different EU vc type (Table 5). Another strain was isolated from the uninoculated control canker and, as expected, it was different from Cp 4.2H (result not shown). The natural canker treated by shooting with Cp 4.2H discs was clearly healing 1 year after inoculation.

We did not observe any phytotoxic effects in the impact area of the pellet of the control (used without mycelial disc) and all wounds healed promptly.

Discussion

Since 1970s, chestnut restoration projects included release of hypovirulent strains to reduce chestnut blight in Europe (Falcini *et al.* 1980; Bisiach *et al.* 1991; Turchetti and Maresi 1988; Heiniger and Rigling 1994; Robin and Heiniger 2001; Milgroom and Cortesi 2004). However, several studies revealed that biocontrol alone was not sufficient to slow down the blight spread because hypovirulence dissemination can be hampered by several factors and seldom correlates with the release of hypovirulent strains (Falcini *et al.* 1980; Bisiach *et al.* 1991; Robin *et al.* 2000, 2010; Milgroom and Cortesi 2004; Mcdonald and Double 2005).

Formulation and delivery of hypovirulent strains received little attention, although we know that an effective formulated biopesticide must be stable over time in order to maintain micro-organism viability until the biologically active ingredient gets in contact with the

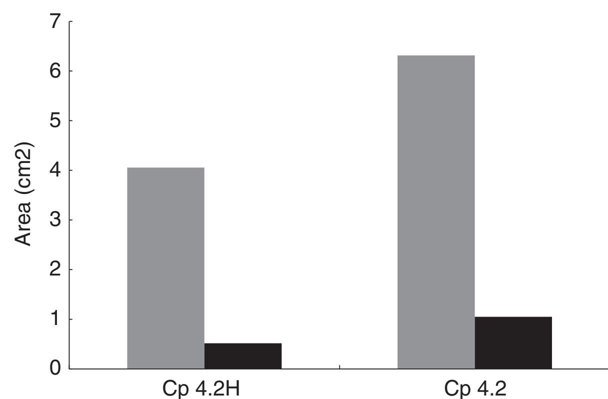

Figure 3 Cankers area measured on *Castanea sativa* stems 4 weeks after shotgun (dark grey) and wound applications (black) of *Cryphonectria parasitica* mycelium in controlled conditions.

Table 5 Characterization of *Cryphonectria parasitica* strains re-isolated from cankers obtained shooting mycelium discs

Sample	Strain	EU vc type	MAT	Fingerprint type	Identification
	Cp 4.2	2	1	B	
	EU-1*	1	2		
	EU-2*	2	1		
2	2·1	13	2	C	nd†
3	3·1	13	2	C	nd
5	5·1	2	2	B	nd
6	6·1	2	2	B	nd
	6·2	2	1	B	Cp 4.2
7	7·1	2	2	B	nd
	7·2	2	2	B	nd
11	11·1	17	1	D	nd
	11·2	17	1	D	nd
	11·3	2	1	B	Cp 4.2
12	12·1	2	1	B	Cp 4.2
	12·2	2	1	B	Cp 4.2
	12·3	2	1	B	Cp 4.2
13	13·1	2	1	B	Cp 4.2
	13·2	2	1	B	Cp 4.2
	13·3	2	1	B	Cp 4.2
15	15·1	2	1	B	Cp 4.2
	15·2	2	1	B	Cp 4.2
16	16·1	2	1	B	Cp 4.2
	16·2	2	2	B	nd

*Reference strains of EU vc types, collection of the Plant Pathology Laboratory, Department of Food, Environmental and Nutritional Sciences (DeFENS), University of Milan.
†Not determined.

pathogen, be user- and environmentally friendly and cost-effective (Montesinos 2003; Glare *et al.* 2012). Therefore, we proposed a new formulation and delivery method for a *C. parasitica* hypovirulent strains to satisfy these requirements.

The *C. parasitica* hypovirulent mycelium was formulated to prepare a laminated hydrophilic dried matrix, from which we obtained mycelial discs. The two coformulants used, PEG400 and HPMC, contributed to an efficient lamination of the mycelium mix, and to *in vitro* stability of the fungus, which had a DT50 of 72 days when stored at 6 ± 1°C. Rehydration of the formulated mycelium might be a critical issue, especially for the hypovirulent strain not forming conidia. Cell death may occur and host infection will not be established (Herrmann and Lesueur 2013). Beker *et al.* (1984) reported that PEG enables water absorption, and at the same time, prevents the loss of cellular material through the reduction of membrane permeability (Beker *et al.* 1984). HPMC has the property of gelation, and unlike methylcellulose used as additive in other biopesticide formulations (Larena *et al.* 2007), its hydration is temperature dependent. We hypothesize that such behaviour could enable embedding of the micro-organism and protection from desiccation, so that revitalization and progressive release of the fungus is easily achieved (Hari *et al.* 2015). Formation of coformulant gel *in situ* also prevents *C. parasitica* wash off, which can be one of the possible causes of biocontrol failure (Milgroom and Cortesi 2004). In our formulation, we preferred a low viscosity-grade HPMC capable of a fast gel formation that allowed for release of the fungus propagules in low moisture environment. Last, but not less important, formulators can manipulate HPMC without concern since it is certified to be safe.

The estimated shelf life (DT50) of our hypovirulent Cp 4.2H strain preparation—2·5 months—was shorter than that of the Cp 4.2 virulent one and of other formulated biofungicides (Larena *et al.* 2007; Kobori *et al.* 2015). We expected this lower viability, because, unlike the other biofungicides based on sporulating fungi, *C. parasitica* hypovirulent strain formulations consist mainly of mycelium known to be less resistant than spores to adverse conditions (Glare *et al.* 2012). However, this limitation can be overcome exploring recent innovative formulations based on zeolites and biopolymers, which increased the stability of *Serratia entomophila* (Glare *et al.* 2012).

The *C. parasitica*-formulated hypovirulent Cp 4.2H strain was delivered *in vivo* using lead-free pellets as carrier, that were shot in the inner bark tissues of excised chestnut stems. The inoculation experiment showed that the fungus from formulated mycelium grew faster and developed significantly more extensive cankers when applied by shooting than by cork-borer method. This result can be associated either with a more stable and protected formulated mycelium or with the type of wound produced by the delivery method. The shot bark tissue had lesion margins less definite than the cork-borer

wound, which might have facilitated the infection of a wider area by the inoculum. To succeed in chestnut blight management, once delivered, *C. parasitica* hypovirulent strains must be bioavailable *in planta* to promptly get in contact with the virulent one for hypovirus transmission, and outcompete resident microflora (Mcdonald and Double 2005).

When the shooting delivery method was tested in field, we also obtained promising results. The recovery rate of Cp 4.2H strain from cankers 1 year after shooting was 37·5%; and all re-isolated strains were virus infected. These results are in agreement with the most successful biocontrol cases reported in the literature (Hogan and Griffin 2002; Milgroom and Cortesi 2004).

Application technologies to treat plant trunks available on the market are quite rare; one is Chemjet Tree Injector (http://www.chemjet.com.au/, accessed July 2016), but this technique is useful for low height applications only. On the contrary, our proposed shooting method for the delivery of hypovirulent *C. parasitica* mycelium discs is suitable also for applications to high-positioned cankers from the ground with no additional positioning costs.

In forest pest management, the balance between the cost and benefit of each measure is critical. While the use of fungicides is justified only in orchards, the biological control by hypovirulence is a valuable alternative both in orchards and in forests, but it must be cost-effective and easy to apply. Milgroom and Cortesi (2004) sustained that intensive treatments can increase the success of chestnut blight control, and preventive applications also gave good results (Willey 1982). Unlike the standard inoculation method, the proposed procedure could be applied to large scale as it is economically sustainable, because the application is faster compared to hand-inoculation. However, it requires highly skilled personnel, that is, good shooters and able to recognize which cankers to inoculate in order to obtain the highest probability of healing. In conclusion, we recognize that the shooting is the most critical point of the procedure, but instructed operators could ultimately apply it to improve the delivery of hypovirulent strains for chestnut blight biocontrol.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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