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

Running title: New chestnut blight biocontrol

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Abstract

Aims: This study aimed to develop a new formulation of *Cryphonectria parasitica* hypovirulent mycelium suitable for long distance applications. *C. 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 disks with polyethylene glycol (PEG) and hydroxypropyl methylcellulose (HPMC), loaded into lead-free pellets used as

carriers to inoculate cankers on chestnut stems by shooting. The formulation of the 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 disks of hypovirulent strain Cp 4.2H were airgun shot into the chestnut bark. *In planta*, Cp 4.2H was recovered in 37% of bark samples taken around the inoculated points one year after the treatment.

Conclusions: We demonstrated that the proposed airgun shooting inoculation method of *C. parasitica* hypovirulent strain formulated as mycelium disks 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.

Keywords: hypovirulence, carrier, bioavailability, HPMC, *Castanea sativa*

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). *C. 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* (Biraghi 1953) and subsequently, the researches proved the existence of hypovirulent strains in these cankers

(Heiniger and Rigling 1994). Hypovirulence is related to *Cryphonectria hypovirus* (CHV), species of dsRNA viruses which parasitize the fungus (Choi and Nuss 1992) and reduce its virulence. The transmission of the hypovirus to the pathogen occurs via anastomosis or asexual spores. Since this discovery, the scientific community recognized the hypovirulence as a potent tool for the disease management; however, there are still difficulties to obtain effective chestnut blight control.

Several papers highlighted the most critical issues in the 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/non-self 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/non-self 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 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, i.e. 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 (Walters 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 programs 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 manually 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 i) developed a formulation of *C. parasitica* suitable for long distance deployment and evaluated it for ii) persistence of the bioactive control agent during conservation, iii) efficacy of a hypovirulent strain delivery, and iv) its survival *in vivo* and in the natural context.

88 **Materials and methods**

89 **Inoculum preparation and formulation**

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

99 The dried mycelium was ground in the ultra centrifugal mill (mod. ZM200 Retsch, Hann, Germany) with
100 0.25 mm sieve, 12-teeth rotor rotating at 2012.4 g. Grinding efficiency (GE) equal to percent ratio of
101 mycelium recovered with respect to mycelium ground, disk preparation and overall yields were assessed.
102 The powder was mixed in mortar with polyethylene glycol (3-4% w/w, PEG 400, ACEF, Fiorenzuola
103 D'Arda, Italy), hydroxypropyl methylcellulose (32-39%, HPMC; Methocel E5; Dow, USA) and water (39-
104 50%) for three min to obtain a homogeneous semisolid and sticky paste, which was laminated to 1.5 mm
105 thickness by a manual twin roll calander, and dried at 25°C for 12 h. The dry films were die-cut into disks of
106 2.5 mm diameter.

107 Technical and biological properties were assessed both for hypovirulent and virulent mycelia on 10
108 randomly selected disks. The technical parameters were: average weight, thickness, diameter of the disks,
109 and biological active ingredient concentration (%). The biological parameters were: viability of the dried and
110 grounded mycelium (not formulated) and formulated mycelium, and fungal concentration in the formulated

disks at t_0. Before formulation, 1 mg of mycelium was suspended in 1 ml of sterile water containing 0.02% Tween80 (Sigma-Aldrich, USA) in an Eppendorf tube, and the tube was vortexed for 30 s. Serial dilutions were streaked on malt extract agar (MEA, Oxoid). The colony-forming units (CFUs) were counted after seven days of incubation and the viability estimated on three replicates was expressed as log (CFUs) mg^{-1} of mycelium. The fungal concentration in the formulated disks at t_0 was estimated as follows: disks were soaked for one 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 disks accurately weighted in a tissue bag (Shandon™ nylon biopsy bags small, Thermo Scientific™, Waltham, MA USA). The bags were placed in a tube of a disintegration test apparatus (DT3, Sotax, Thun, CH) oscillating at 25 strokes per minute 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 percent ratio between the final weight of the dry mass and the initial weight of the disks.

Shelf life of the mycelium disks

Mycelium disks were stored in refrigerator at 6±1 °C. Their shelf life was estimated every month for six months as fungal concentration of each disk, 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 to hypovirulence the virus-free isogenic strain, 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, 2004; Davis, 2005).

134 **Mycelium disks delivery method**

135 Each *C. parasitica* mycelium disk was glued with an aqueous solution of povidone (15% w/w, Kollidon 30,
136 BASF, Ludwigshafen, Germany) within the concavity of a hollow point lead-free 4.5 mm diameter pellet
137 (Crosman corp., Bloomfield, USA). The pellet, loaded either with the virulent or the hypovirulent
138 formulation, was shot from a 10 m distance in the chestnut bark with a Weihrauch HW977 airgun lever
139 action rifle cal. 4.5 mm, so that the pellet entered 1-1.5 cm depth within the bark.

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

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

149 The delivery method was also assessed in the field, inoculating 20 chestnut stems at two to five meter height,
150 shooting pellets loaded with mycelium disks of Cp 4.2H hypovirulent strain only. Hand wound inoculations
151 were carried out as previously described on the same number of randomly chosen stems (N= 20). In addition,
152 one natural canker was treated shooting the pellets close to the margin around the canker. As control, stems
153 were inoculated with pellets not loaded with the mycelium. One year after the inoculation, 16 randomly
154 chosen bark wounds were measured and analyzed for the presence of the Cp 4.2H released strain, then bark
155 samples were taken around the point of entrance of the pellet to assess the presence of the hypovirulent
156 strain**.** In addition, in the same area we sampled an untreated canker (un-inoculated control) to isolate *C.*

157 *parasitica* strains naturally occurring in the field. Isolation and purification were performed as previously
158 described (Cortesi *et al*. 1996).

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

165 **Statistical analysis**

166 Mycelium viability and canker area data were analyzed by t-Student test using R software, version R3.0.2.
167 (R Core Team, 2013).

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

173

174 **Results**

175 **Mycelium formulation**

176 Mycelium of hypovirulent and virulent strains grown in liquid culture was filtered, dried and finely ground.
177 The powder was added with excipients for the preparation of disks. The yields after grinding and disk
178 preparation steps ranged between 73% and 85% for Cp 4.2H and Cp 4.2, respectively, and the overall

179 formulation yields were 58% and 72% for Cp 4.2H and Cp 4.2, respectively (Table 1). The mycelium
180 concentration in the formulated disks was 30% (w/dry w) with a titer ranging between 3.8 log (CFUs mg^{-1})
181 and 4.1 log (CFUs mg^{-1}) (Table 2). The formulation did not affect significantly the fungus viability as
182 confirmed from the t Student test for both strains: P = 0.1394 and P = 0.5172, respectively (Table 3). In
183 order to verify the complete release of the strain following hydration, the weight loss of the disks was
184 evaluated *in vitro* following immersion in water. The relatively high water solubility and low viscosity of the
185 cellulosic polymer used for the formulation allowed complete disks erosion within 60 min (Fig. 1).

186 **Formulated mycelium shelf life**

187 The number of viable propagules of Cp 4.2H disks after 6 months storage at 6±1 °C declined from 3.8 CFUs
188 mg^{-1} to 2.1 CFUs mg^{-1}, whereas for Cp 4.2 the reduction was lower from 4.1 CFUs mg^{-1} to 2.9 CFUs mg^{-1}
189 (Table 4). The estimated DT50 for the two strains were 72 and 85 days, respectively (Table 4; Fig. 2). After
190 6 months of storage, we re-isolated the mycelia from formulated disks to check for hypovirulence, the lack of
191 contamination and the strain morphological and genotypic characters. We did not detect any contaminants in
192 the formulated disks. The morphological characters, the presence of the CHV-1 hypovirus in Cp 4.2H, the
193 mating type, *MAT*-1, and the fingerprint profile of the two strains, before and after the formulation, were
194 identical (results not shown).

195 ## Delivery method

196 We assessed the bioavailability of the mycelium delivered by shotgun in two experiments, the first *ex vivo* in
197 controlled conditions, the other in a small-scale field trial. In controlled conditions, all inoculations
198 originated cankers, independently of the method used. However, by shotgun delivery, the area of the cankers
199 were respectively six- to eightfold bigger than those originated by the standard cork-borer wound
200 inoculation, both for the hypovirulent Cp 4.2H (P=4.199E-3) and for the virulent Cp 4.2 formulations
201 (P=9.182E-9; Fig. 3). In the field, where we delivered only Cp 4.2H in randomly chosen trees, the cankers

developed following shotgun inoculation were on average 4.83±0,29 cm^2, not significantly different from those obtained by hand wound inoculation, which averaged 3.61±1,46 cm^2. We isolated 20 *C. parasitica* strains from 10 out of 16 bark samples taken from the shotgun cankers. All purified strains were analyzed for the mating type, vegetative compatibility group and fingerprint profile. We identified 11 strains from 6 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 un-inoculated 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 disks was clearly healing one year after inoculation.

We did not observe any phytotoxic effects in the area of impact of the pellet of the control (used without mycelial disk) 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 1991; Heiniger and Rigling 1994; Robin and Heiniger 2001; Milgroom and Cortesi 2004). However, several studies unraveled 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; Milgroom and Cortesi 2004; Mcdonald and Double 2005; Robin *et al.* 2010).

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 microorganism viability until the biologically active ingredient gets in contact with the pathogen, and 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 disks. 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 non-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 behavior could enable embedding of the microorganism 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).

249 The *C. parasitica* formulated hypovirulent Cp 4.2H strain was delivered *in vivo* using lead-free pellets as
250 carrier, that were shot in the inner bark tissues of excised chestnut stumps. The inoculation experiment
251 showed that the fungus from formulated mycelium grew faster and developed significantly more extensive
252 cankers when applied by shooting than by cork-borer method. This result can be associated either to a more
253 stable and protected formulated mycelium or to the type of wound produced by the delivery method. The
254 shot bark tissue had lesion margins less definite than the cork-borer wound, which might have facilitated the
255 infection of a wider area by the inoculum. To succeed in chestnut blight management, once delivered,
256 *C.parasitica* hypovirulent strains must be bioavailable *in planta* to promptly get in contact with the virulent
257 one for hypovirus transmission, and outcompete resident microflora (Mc Donald and Double 2005).

258 When the shooting delivery method was tested in field, we also obtained promising results. The recovery rate
259 of Cp 4.2H strain from cankers one year after shooting was 37.5%; and all re-isolated strains were virus
260 infected. These results are in agreement with the most successful biocontrol cases reported in the literature
261 (Hogan and Griffin 2002; Milgroom and Cortesi 2004).

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

267 In forest pest management, the balance between the cost and benefit of each measure is critical. While the
268 use of fungicides is justified only in orchards, the biological control by hypovirulence is a valuable
269 alternative both in orchards and in forests, but it must be cost-effective and easy to apply. Milgroom and
270 Cortesi (2004) sustained that intensive treatments can increase the success of chestnut blight control
271 (Milgroom and Cortesi 2004) and preventive applications also gave good results (Willey 1982). Unlike the
272 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, i.e. 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 more 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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375 **List of tables**

376 **Table 1** Yields of formulated *Cryphonectria parasitica* mycelium

	Yield (%)	
Strain	Cp 4.2H*	Cp 4.2
Grinding efficacy	79.4	83.0
Disk preparation	73.3	85.7
Overall formulation	58.2	71.2

377 *CHV1 infected strain

378

379 **Table 2** Properties of formulated mycelium disks 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 CFUs mg^{-1}) at t_0	3.8±1.2	4.1±1.36

380 *N = 10 disks; **CHV1 infected strain

381

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

	Concentration (log CFUs mg^{-1})				
Strain	Pre-formulation*	Post-formulation**	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

383 *Pre-formulation refers to mycelium dried and ground; **Post-formulation refers to mycelium formulated as
384 disks.

385

386 **Table 4** Model parameters and DT50 estimated values for disks of *Cryphonectria parasitica* formulated
387 mycelium

Strain	$CFUs^{-mg}$ 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

388

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

390 mycelium disks

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
	11.1	17	1	D	nd
11	11.2	17	1	D	nd
	11.3	2	1	B	Cp 4.2
	12.1	2	1	B	Cp 4.2
12	12.2	2	1	B	Cp 4.2
	12.3	2	1	B	Cp 4.2
	13.1	2	1	B	Cp 4.2
13	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

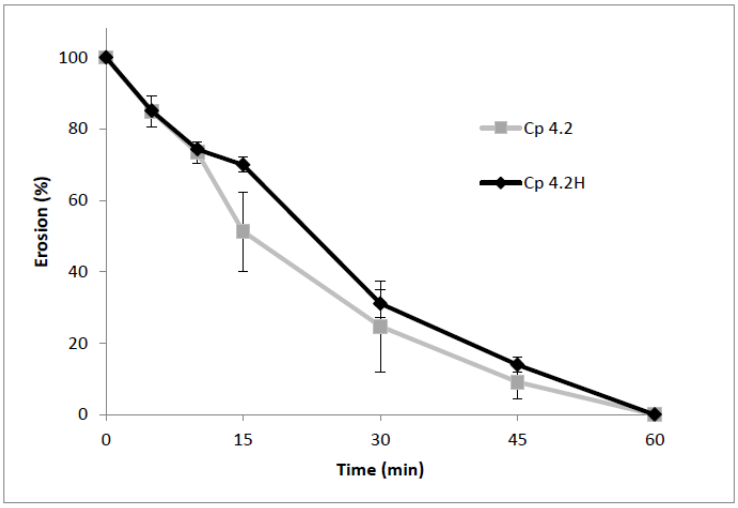
16	15.2	2	1	B	Cp 4.2
	16.1	2	1	B	Cp 4.2
	16.2	2	2	B	nd

391 *Reference strains of EU vc types, collection of the Plant Pathology Laboratory, Department of Food,

392 Environmental and Nutritional Sciences (DeFENS), University of Milan; ** Not determined

393

394 **List of Figures**



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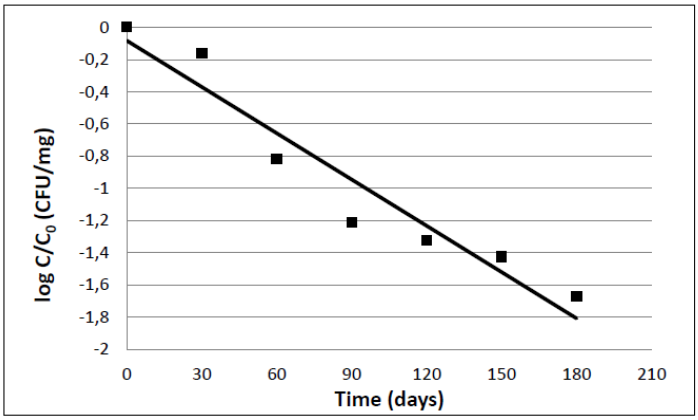
396 **Fig. 1** Water erosion profiles of *Cryphonectria parasitica* mycelium formulated disks. Bars indicate

397 standard errors.



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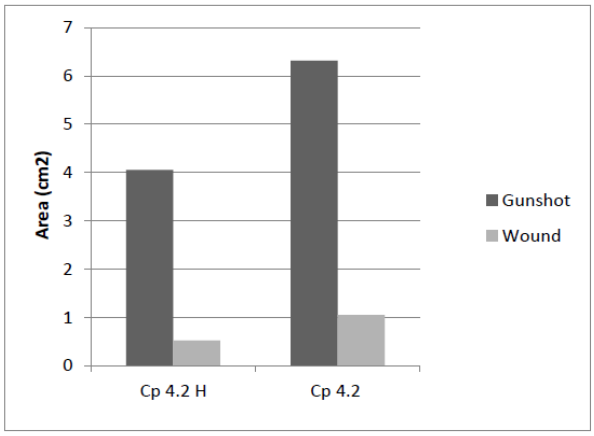
399 **Fig. 2** First order kinetic model for shelf life of *Cryphonectria parasitica* hypovirulent formulated

400 mycelium Cp 4.2H.



401

402 **Fig. 3** Cankers area measured on *Castanea sativa* stems 4 weeks after shotgun and wound applications of

403 *Cryphonectria parasitica* mycelium in controlled conditions.