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

Running title: New chestnut blight biocontrol

Andrea Kunova¹, Cristina Pizzatti¹, Matteo Cerea², Andrea Gazzaniga² and Paolo Cortesi¹*

¹ Department of Food, Environmental and Nutritional Sciences, University of Milan, Milan, Italy
² Department of Pharmaceutical Sciences, University of Milan, Milan, Italy

* Correspondence: Paolo Cortesi, Department of Food, Environmental and Nutritional Sciences, University of Milan, Via Celoria 2, 20133 Milan, Italy, paolo.cortesi@unimi.it

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](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

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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

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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.

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and delivery method of *Cryphonectria parasitica* for biological control of chestnut
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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 (PDB; Oxoid, Hampshire, UK) supplemented with tetracycline (50 mg l⁻¹). The flasks were shaken at 100 rpm with an orbital shaker (mod. M201-OR, MPM Instruments, Bernareggio, Italy) for seven 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 (GE) equal to percent ratio of mycelium recovered with respect to mycelium ground, disk 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; Dow, USA) and water (39-50%) for three min to obtain a homogeneous semisolid and sticky paste, which was laminated to 1.5 mm thickness by a manual twin roll calander, and dried at 25°C for 12 h. The dry films were die-cut into disks of 2.5 mm diameter.

Technical and biological properties were assessed both for hypovirulent and virulent mycelia on 10 randomly selected disks. The technical parameters were: average weight, thickness, diameter of the disks, and biological active ingredient concentration (%). The biological parameters were: viability of the dried and grounded mycelium (not formulated) and formulated mycelium, and fungal concentration in the formulated
disks at t₀. 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 t₀ 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).
Mycelium disks delivery method

Each *C. parasitica* mycelium disk 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, 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, so that the pellet entered 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 stumps 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 stump was covered with a layer of paraffin, and the bottom was dipped in five 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 applying the formula for an ellipse (*πLW*/4).

The delivery method was also assessed in the field, inoculating 20 chestnut stems at two to five meter height, shooting pellets loaded with mycelium disks 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 analyzed for the presence of the *Cp* 4.2H released strain, then bark samples were taken around the point of entrance of the pellet to assess the presence of the hypovirulent strain. In addition, in the same area we sampled an untreated canker (un-inoculated control) to isolate *C.*

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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. 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 analyzed by t-Student test using R software, version 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: $t = l/k \ln(C_0/C_t)$ 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 data.

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 disks. The yields after grinding and disk preparation steps ranged between 73% and 85% for Cp 4.2H and Cp 4.2, respectively, and the overall

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formulation yields were 58% and 72% for Cp 4.2H and Cp 4.2, respectively (Table 1). The mycelium concentration in the formulated disks was 30% (w/dry w) with a titer ranging between 3.8 log (CFUs mg\(^{-1}\)) and 4.1 log (CFUs mg\(^{-1}\)) (Table 2). The formulation did not affect significantly the fungus viability as confirmed from the t Student test for both strains: \(P = 0.1394\) and \(P = 0.5172\), respectively (Table 3). In order to verify the complete release of the strain following hydration, the weight loss of the disks 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 disks erosion within 60 min (Fig. 1).

**Formulated mycelium shelf life**

The number of viable propagules of Cp 4.2H disks after 6 months storage at 6±1 °C declined from 3.8 CFUs 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\(^{-1}\) (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 disks to check for hypovirulence, the lack of contamination and the strain morphological and genotypic characters. We did not detect any contaminants in the formulated disks. 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 area 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 (\(P=4.199E-3\)) and for the virulent Cp 4.2 formulations (\(P=9.182E-9\); 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 cm², not significantly different from those obtained by hand wound inoculation, which averaged 3.61±1.46 cm². 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 biosticide 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).
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 stumps. 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 to a more stable and protected formulated mycelium or to 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 (Mc Donald 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 one 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 is Chemjet Tree Injector ([http://www.chemjet.com.au/](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 disks is suitable also for long distance applications to high positioned cankers 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 (Milgroom and Cortesi 2004) 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, 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.

**Acknowledgements**

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**Conflict of interest**

The authors declare that there is no conflict of interest.

**References**


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## List of tables

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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cp 4.2H*</td>
<td>Cp 4.2</td>
</tr>
<tr>
<td>Grinding efficacy</td>
<td>79.4</td>
</tr>
<tr>
<td>Disk preparation</td>
<td>73.3</td>
</tr>
<tr>
<td>Overall formulation</td>
<td>58.2</td>
</tr>
</tbody>
</table>

*CHV1 infected strain

### Table 2 Properties of formulated mycelium disks of *Cryphonectria parasitica*

<table>
<thead>
<tr>
<th>Property</th>
<th>Cp 4.2H**</th>
<th>Cp 4.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (mg)</td>
<td>5.73±0.72</td>
<td>6.82±0.79</td>
</tr>
<tr>
<td>Thickness (mm)</td>
<td>1.23±0.29</td>
<td>1.22±0.07</td>
</tr>
<tr>
<td>Diameter (mm)</td>
<td>2.45±0.01</td>
<td>2.48±0.07</td>
</tr>
<tr>
<td>Mycelium content (mg)</td>
<td>1.7</td>
<td>2.0</td>
</tr>
<tr>
<td>Viability (log CFUs mg⁻¹) at t₀</td>
<td>3.8±1.2</td>
<td>4.1±1.36</td>
</tr>
</tbody>
</table>

*N = 10 disks; **CHV1 infected strain

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which has been accepted for publication at http://onlinelibrary.wiley.com/doi/10.1111/jam.13328/full. This article may be used for non-commercial purposes in accordance with Wiley Terms and Conditions for Self-Archiving.*
Table 3 Viability of *Cryphonectria parasitica* mycelium before and after formulation at $t_0$

<table>
<thead>
<tr>
<th>Strain</th>
<th>Pre-formulation*</th>
<th>Post-formulation**</th>
<th>T value</th>
<th>P value</th>
<th>CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cp 4.2H</td>
<td>1.686</td>
<td>1.188</td>
<td>2.3893</td>
<td>0.1394</td>
<td>-0.398; 1.394</td>
</tr>
<tr>
<td>Cp 4.2</td>
<td>1.775</td>
<td>1.674</td>
<td>0.7797</td>
<td>0.5172</td>
<td>-0.459; 0.662</td>
</tr>
</tbody>
</table>

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

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

<table>
<thead>
<tr>
<th>Strain</th>
<th>CFUs$^{\text{disc}}$ at $t_0$</th>
<th>CFUs$^{\text{disc}}$ at endpoint</th>
<th>k</th>
<th>$R^2$</th>
<th>DT50 (Days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cp 4.2H</td>
<td>3.8</td>
<td>2.1</td>
<td>-0.0096</td>
<td>0.9263</td>
<td>72.2</td>
</tr>
<tr>
<td>Cp 4.2</td>
<td>4.1</td>
<td>2.9</td>
<td>-0.0081</td>
<td>0.5915</td>
<td>85.6</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Sample</th>
<th>Strain</th>
<th>EU vc type</th>
<th>MAT</th>
<th>Fingerprint type</th>
<th>Identification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cp 4.2</td>
<td>2</td>
<td>1</td>
<td>B</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EU-1*</td>
<td>1</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>EU-2*</td>
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*This is the pre-peer reviewed version of the following article:
This is the pre-peer reviewed version of the following article:
Fig. 2 First order kinetic model for shelf life of *Cryphonectria parasitica* hypovirulent formulated mycelium Cp 4.2H.

Fig. 3 Cankers area measured on *Castanea sativa* stems 4 weeks after shotgun and wound applications of *Cryphonectria parasitica* mycelium in controlled conditions.

"This is the pre-peer reviewed version of the following article: