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Hail-induced infections of the chestnut blight pathogen *Cryphonectria parasitica* depend on wound size and may lead to severe diebacks

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ABSTRACT

This study combined phytosanitary surveys, laboratory analyses and mathematical modelling to show how hail-induced wounds can foster the infections of the blight pathogen *Cryphonectria parasitica*, locally associated with extensive dieback of chestnut (*Castanea sativa*). Orchards and coppices located within and outside the assessed dieback area in a single location in the North West of Italy were inspected to appraise the abundance of hail-induced wounds and *C. parasitica* infections. The incidence of *C. parasitica* was significantly higher within the dieback area compared to outside (92% *vs.* 60%; $P < 0.05$). Hail-induced wounds were observed on small branches and shoots of all trees sampled within the dieback area, whereas they were less abundant outside (20% of trees), suggesting either that the dieback was directly associated with the injuries caused by the hailstorms or that those injuries may have facilitated infections of *C. parasitica*. Isolations conducted on 359 branches and shoots showed that hail-induced wounds served as infection courts for *C. parasitica* and that infections depended on the size rather than on the number of hail wounds. We fitted a logistic model showing that hail-induced wounds whose perimeter was larger than 66 mm were at particular risk of *C. parasitica* infection. A newly designed geometrical-based model (GAHW) is proposed to relate hailstones size, hail wound perimeter and the risk of infection. We established that hail-induced wounds are entry points for virulent and hypovirulent strains of *C. parasitica*, since 6.5% of isolates were infected by Cryphonectria hypovirus-1.

KEYWORDS

chestnut blight, *Castanea*, climate change, dieback, epidemiology, hypovirulence, modelling, risk assessment

INTRODUCTION

The European or sweet chestnut (*Castanea sativa* Mill.), hereafter referred to as chestnut, is a multipurpose tree species valued for timber, nuts and for other ecosystem services (Bounous and Torello Marinoni 2005). In Southern Europe, chestnut has historically served as a key staple food and firewood source for people living in mountain areas (Conedera et al. 2004a, b). Nowadays, chestnut not only supports a niche yet important demand from the food and wood industry, but plays an important role in soil protection, landscape conservation, biodiversity conservation and for the provision of recreational areas and the production of secondary products, including honey, tannins and edible mushrooms (Bounous and Torello Marinoni 2005; Vogt et al. 2006).

In Europe, chestnut has been challenged by several diseases, including ink disease caused by the fungal-like organisms *Phytophthora cambivora* (Petri) Buisman and *P. cinnamomi* Rands, and chestnut blight caused by the fungus *Cryphonectria parasitica* (Murrill) M.E. Barr, which were responsible for severe epidemics in the 19th and 20th centuries, respectively (Gonthier and Robin 2020). More recently, chestnut has been threatened by the fungal nut rot and canker agent *Gnomoniopsis castaneae* G. Tamietti and by the Asian gall wasp *Dryocosmus kuriphilus* Yasumatsu (Hymenoptera Cynipidae), an invasive insect inducing the formation of galls (Avtzis et al. 2019; Lione et al. 2019; Ôtake 1980).

Both threats first emerged in the early 2000s in the North West of Italy, and they have been subsequently reported throughout most of the distribution range of chestnut in Europe (Avtzis et al. 2019; Lione et al. 2016, 2019).

In the North West of Italy, like in many other European areas, chestnut blight has long been recognized as highly prevalent both in orchards and in coppices, although starting from the 1970s disease severity gradually decreased, especially in the oldest disease hot spots where most of the cankers were observed to heal (Gonthier and Robin 2020). The observed mitigation of chestnut blight symptoms was the result of a biological phenomenon known as hypovirulence, which is mainly due to the occurrence and spread in Europe of the virus Cryphonectria hypovirus-1 (CHV1) that is able to infect *C. parasitica*, thereby reducing its aggressiveness (Milgroom and Cortesi 2004; Rigling and Prospero 2018).

In between the late 2000s and the early 2010s, chestnut growers and forest owners reported the occurrence of severe diebacks affecting both chestnut orchards and coppices across some areas of the province of Cuneo, North West of Italy. Since the Asian gall wasp had been heavily infesting that region starting from 2001 (Quacchia et al. 2008), it was firstly argued that the pest could have been the main causal agent of the dramatic decline observed across the chestnut stands. Preliminary surveys pointed out that a prominent role in the aetiology of the dieback might have been played by the blight, whose incidence and severity seemed abnormally high. However, the above surveys also led to the observation that trees had been recently challenged by intense hailstorms because of the presence of hail wounds on branches and shoots and of holes and ripping on leaves. These in-field observations were confirmed by reports of local farmers that claimed an increased abundance and severity of hailstorms events. It is worth noting that *C. parasitica* is a necrotrophic pathogen requiring fresh wounds or growth cracks in the bark to penetrate into the host tissues (Rigling and Prospero 2018). Therefore, hail wounds may have represented infection courts for the pathogen thus triggering blight-related diebacks as documented for other pathogens associated with blight and cankers, including the fungus *Sphaeropsis sapinea* (Fr.) Dyko & B. Sutton on pines and the bacterium *Erwinia amylovora* (Burrill) Winslow, Broadhurst, Buchanan, Krumwiede, Rogers & Smith on apple trees (Bobev and Deckers 1999; Lanthier 2011; Smith et al. 2002; Zwolinski et al. 1995). Hailstorms have been previously suggested to enhance the occurrence of *C. parasitica* infections (EFSA PLH Panel 2014; Turchetti et al. 2010), but there is a complete lack of data and of quantitative information about the association between hail wounds on trees, the infection process and the development of blight and cankers.

Therefore, the goals of this study were: i) to assess whether infection by *C. parasitica* may depend on the number, shape or size of hail wounds and dimension of hail of hailstones, and to model the risk of infection accordingly, and ii) to determine if infection through hail wounds may occur indistinctively by virus-free and virus-infected strains of the pathogen.

MATERIALS AND METHODS

Study area and sites

Diebacks of chestnuts were reported by local authorities from the second half of 2000s in the Province of Cuneo, Piedmont Region, North West of Italy, and in particular in the municipality of Peveragno (lat. 44° 19' 44.6''; long. 7° 37' 2.8''; elevation 588 m a.s.l.). Preliminary surveys in the area were performed in June 2012. Diebacks involved both chestnut orchards and coppices growing on hills located W-SW from the town of Peveragno and covering an approximate surface of 612 ha distributed from 600 to 1000 m a.s.l. Based on their hillslope position (Miller and Schaetzl 2015), orchards were mostly located in the toeslope and lower footslope portions, while coppices covered the higher footslope, backslope and shoulder of the hill. Trees in orchards were characterized by a mean diameter at breast height (DBH) of approximately 53 cm, while the DBH of stems in coppices ranged from 25 to 35 cm depending on the rotation time. Soils in the area are classified as Typic Hapludalf (IPLA - Regione Piemonte 2007). The mean annual temperature (years 1988-2012) is 10.9°C and the mean precipitation is 1048 mm (ARPA - Piemonte, 2019).

One peculiar feature of the dieback phenomenon was that a relatively sharp separation occurred between the dieback and the surrounding area (Fig. 1 and 2), which were similar in terms of tree species composition and stand structure. Hence, five study sites within the dieback area (w sites) and three study sites outside the dieback area (o sites) were selected across the hills surrounding the town of Peveragno (Fig. 2). The distance between w sites ranged from 544 m to 1954 m, with an average of 1157 m, while o sites were separated by a distance ranging from 1109 m to 2391 m, with an average of 1860 m. Each study site was established to cover a surface of about 1.5 ha encompassing both orchard and coppice.

Field inspections and samplings

A first survey was conducted on June 5th, 2012 across the eight study sites. Crown transparency was estimated as a proxy of the degree of tree decline associated with diebacks, since this parameter is related to tree vitality, as documented for chestnut (Gehring et al. 2018), as well as for other tree species (Giordano et al. 2009; Jung et al. 2000; Lione et al. 2012). Crown transparency is expressed for each tree as a score appraising the leaves loss (in %) in relation to an ideal status where defoliation is absent (Müller and Stierlin 1990). In each study site, the crowns of 15 chestnuts located along a linear transect were visually inspected and scores were assigned with the aid of a set of reference plates depicting different levels of transparency (Lione et al. 2012; Müller and Stierlin 1990), as reported below. Two viewpoints per tree were established in positions allowing a complete overview of the crown, along perpendicular compass directions and at a distance equal to the height of the tree (Durrant et al. 2006; Müller and Stierlin 1990). Crown transparency scores ranging from 0% to 100%, with 5% unit increments, were assigned (Lione et al. 2012). As recommended in Durrant et al. (2006), two experienced operators performed and discussed the outcomes of the assessment and a consensus was reached.

The presence/absence of blight symptoms, galls of *D. kuriphilus* and hail-induced wounds was checked at the tree level as described below. In the field, up to 4 branches per tree were randomly selected and their terminal portion of approximately 1.50 m was excised and visually inspected. If at least one of these terminal portions displayed symptoms or signs of blight, galls, or hail wounds, the corresponding tree was classified as positive to the disease, the pest, or the damage, respectively. The distinction between galls and hail-induced wounds was performed visually, based on their clearly distinguishable morphology (Supplementary file S1).

Since high levels of crown transparency and the concomitant presence of both galls of *D. kuriphilus* and hail-induced wounds were observed only in sites located within the dieback area, while hail-induced wounds were significantly less abundant outside the dieback area (see results), samplings aimed at further contrasting factors potentially inciting diebacks were conducted exclusively in dieback sites during a second survey carried out on November 12th, 2012. Branches and shoots of chestnut were randomly collected along linear transects established across these study sites. The experiment was initially designed to include 400 branches and shoots with an approximate length of 1.5 m, 200 with blight symptoms and 200 without blight symptoms (i.e. asymptomatic). However, given the high abundance of blight symptoms in the area (see results), symptomatic branches and shoots were slightly oversampled, while asymptomatic branches and shoots were less abundant than expected. Hence, the final sample included 212 branches and shoots with blight symptoms and 147 asymptomatic branches and shoots. All 359 branches and shoots were taken to the laboratory for further analyses.

For both samplings, the distinction between symptomatic and asymptomatic branches and shoots was visually performed based on the presence, or not, of at least one among the typical chestnut blight symptoms described in Prospero and Rigling (2013). Galls of *D. kuriphilus* were detected as reported in Lione et al*.* (2016), while hail wounds were identified based on Costello (2014) and Schubert (1991).

Laboratory observations, isolations and molecular analyses

The following variables were assessed in the laboratory for all 359 chestnut branches and shoots: diameter (mm) in close proximity of the cutting surface, length (cm), number of galls of *D. kuriphilus* and number of hail wounds. For each hail wound, its length (mm) and width (mm) were measured and its association or not with visible blight symptoms was recorded by examining the wound surface and contour at the dissecting microscope (40X magnification). Depending on the extension of the length to measure, either a measuring tape graduated in mm or a calliper were used. To confirm that symptoms observed on branches or shoots were indeed caused by *C. parasitica*, *in vitro* isolations were performed from one randomly selected symptomatic hail wound from 60% of symptomatic branches and shoots (127 out of 212). Five subcortical wood samples (3 x 2 mm) were removed from cankers, surface disinfected using 2% sodium hypochlorite for 5 min and rinsed in sterile water. Subsequently, they were placed in 90-mm Petri plates containing potato dextrose agar (PDA – Potato Dextrose Agar 39 g/liter) amended with citric acid (0.5 g/liter). One *C. parasitica* isolate per canker was randomly selected for further analyses. Attempts of isolation were also conducted from 60% of asymptomatic branches and shoots (85 out of 147), by plating randomly collected pieces of wood (3 x 2 mm) as previously described. Pathogen identification was performed based on macro- and micromorphological features of colonies and fruiting structures (EPPO 2005).

To determine if infection through hail wounds had occurred by either virus-free or virus-infected strains of the pathogen, both morphological and molecular diagnostic criteria were used. All *C. parasitica* isolates were grouped in the following three morphological classes: i) virulent, cream-colored mycelium with abundant orange pycnidia, often scattered within concentric rings (orange culture morphology); ii) hypovirulent, white mycelium and few, large pycnidia (white culture morphology); and iii) intermediate, whitish-cream mycelium with pycnidia distributed over the entire colony (intermediate culture morphology) (Bonifacio and Turchetti

1973; Grente and Sauret 1969; Turchetti 1978). Subsequently, all isolates were tested for the presence of Cryphonectria hypovirus-1 (CHV-1) by a newly developed TaqMan®-based real-time PCR assay.

A liquid culture for each isolate was prepared by transferring 4 to 6 plugs of mycelium from an actively growing solid medium culture in 250 ml flasks containing 100 ml Difco potato dextrose broth (PDB, Difco, U.S.). Flasks were incubated for 4 days at room temperature. At the end of the growing period, the medium was removed and the mycelium lyophilized for 24 hours. Total RNA was extracted from approximately 100 mg mycelial powder through the Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, U.S.) following manufacturer's instructions. Complementary DNA (cDNA) was synthesized from total RNA with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, U.S.) following manufacturer's instructions. Subsequently, the TaqMan®-based real-time PCR assay was performed by using 4 primers, CPTub-RealF/CPTub-RealRev for *C. parasitica* β-tubulin gene and CHV1-REalF/CHV1-RealRev for Cryphonectria hypovirus-1 (CHV-1), and two TaqMan® probes (Table 1). The real-time cycling protocol consisted of 95°C activation step for 3 min, and 39 cycles, with each cycle consisting of 95°C for 10 min and 60°C for 30 s. Reactions were carried out using a CFX Connect Real-time PCR detection system (Bio-Rad, U.S.) equipped with FAM reading channel. The TaqMan® assay was carried out in duplicate for each *C. parasitica* isolate; one reference *C. parasitica* CHV-1-positive isolate and one *C. parasitica* CHV-1-negative isolate were included in the assay.

Reconstruction of the occurrence of hailstorm events

The geographic area of North West of Italy where sampling sites were located is not endowed with an official monitoring system recording hailstorms and no quantitative data or measures about position, extension and frequency of the events, as well as about the size of hailstones are available (Baldi et al. 2014; Punge and Kunz 2016). Hence, the reconstruction of hailstorms was performed through event-based and non-systematic reports (Punge and Kunz 2016) from 6 people including local farmers, forest owners and technicians of agricultural entrepreneurs associations (i.e. COLDIRETTI – Cuneo). During the interview, the date of the hailstorms events and the approximate size of the hailstones were asked. When written reports of damages related to hailstorms were available, hailstones diameter was estimated based on the threshold indicated in

Baldi et al. (2014) and in the ANELFA scale relating the hail size to the expected damage to vegetation, people, vehicles, structures and infrastructures (Dessens et al. 2007). The same information was sought through a screening of local newspapers. A further investigation was carried out by querying available meteorological and climatological datasets including the European Severe Weather Database (ESWD) provided by the European Severe Storms Laboratory (ESSL) (Dotzek et al. 2009), the European Climate Assessment & Dataset (Van Den Besselaar et al. 2015), the Storm Report Meteonetwork Dataset (Associazione ONLUS MeteoNetwork 2019), the Meteorologic and Idrologic Database of the Regional Agency for Environmental Protection (ARPA) of Piedmont (ARPA Piemonte 2020). The queries were conducted by extracting all available reports of severe hailstorms occurred throughout2011 and 2012 until November 12th across the study area.

Statistical analyses and modelling

Data from the first survey were analyzed as described below. The average score of crown transparency and the incidence of trees positive to blight symptoms, galls of *D. kuriphilus* and hail-induced wounds (calculated as the ratio between the number of positive trees and the number of sampled trees, in %) were compared between sites located within and outside the dieback area. Comparisons were performed by fitting conditional inference tree models based on the unbiased recursive partitioning algorithm described in Hothorn and Zeileis (2015) and Hothorn et al. (2006). The above algorithm was run by holding its default parameters, including the Bonferroni *P*-value correction for multiple comparisons (Crawley 2013). Conditional inference tree models were fitted by setting both the site and its location either within or outside the dieback area as categorical predictors. The empirical cumulative distribution functions (ECDFs) were fitted to the crown transparency scores assigned to chestnut trees in sites within and outside the dieback area (Crawley 2013).

Data from the second survey were analyzed as follows. For modelling purposes, the shape of hail-induced wounds on branches and shoots of chestnut was approximated by an ellipse (Costello 2014; Schubert 1991) with major and minor axes coincident with the length (L, in mm) and width (W, in mm) measured in the laboratory. Hence, the hail wound surface (S, in mm^2) was calculated with the standard equation (Equation

1) while the perimeter (p, in mm) was approximated by using the Ramanujan's equation (Equation 2) reported in Villarino (2006). To account for shape variations, ellipse eccentricity (e) was calculated with the classical equation (Equation 3) in order to discriminate between circular (e = 0) and stretched elliptical hail wounds (e $\rightarrow$ 1).

$$S = \frac{\pi}{4}LW \qquad \text{(Equation 1)}$$

$$p = \pi\left[\frac{L}{2} + \frac{W}{2} + \frac{3\left(\frac{L}{2}-\frac{W}{2}\right)^2}{10\left(\frac{L}{2}+\frac{W}{2}\right)+\sqrt{\left(\frac{L}{2}\right)^2+\frac{7}{2}LW+\left(\frac{W}{2}\right)^2}}\right] \qquad \text{(Equation 2)}$$

$$e = \frac{\sqrt{\left(\frac{L}{2}\right)^2-\left(\frac{W}{2}\right)^2}}{\frac{L}{2}} \qquad \text{(Equation 3)}$$

By using the same conditional inference tree models described above, symptomatic and asymptomatic branches and shoots were contrasted to compare their average number of hail wounds and galls. In order to prevent any potential bias, contrasts were performed not only on hail wounds (HWC) and galls counts (GC), but also on their ratio to the length of branch or shoot (i.e. HWCL hail wounds/cm and GCL galls/cm). The average values of the variables related to wound shape and dimension (i.e. L, W, S, p, e) were compared between symptomatic and asymptomatic branches and shoots, and between hail wounds associated with blight symptoms or not. The latter comparison was performed both on all wounds and on the subset of wounds present exclusively on symptomatic branches and shoots. In addition, ECDFs were fitted to the number, average values of L, W, S, p, and e of hail wounds and to the number of galls of 212 symptomatic and 147 asymptomatic branches and shoots, whose histograms were derived as well (Crawley 2013).

Based on the results of the previous analyses, binary logistic regressions (Hosmer and Lemeshow 1989) were fitted to model the probability of infection by *C. parasitica* through hail-induced wounds depending on their shape and dimension. The response variable was coded as 1 or 0 for each hail wound associated or not with blight symptoms. The corresponding values of L, W, p, S and e were used as single predictors to fit binary logistic regression models M_L, M_W, M_p, M_S, and M_e, respectively, on the data gathered from 12228 hail wounds (see results). Models' β and β_0 coefficients were calculated along with their related Wald's test *P*-

value (Crawley 2013; Hosmer and Lemeshow 1989). M_L, M_W, M_p, M_S, and M_e were compared to the null-model M_0 by testing their overall significance with the likelihood ratio test (Crawley 2013; Hosmer and Lemeshow 1989) and by contrasting the associated Akaike information criterion (AIC) and AIC weight (AICw) (Grueber et al. 2011; Wagenmakers and Farrell 2004). The 95% confidence intervals of the probability of infection by *C. parasitica* predicted by the above binary logistic regression models were calculated with the algorithm proposed by Heiberger and Holland (2015). The sample size adequacy for model fitting was checked by calculating the EPV index (number of events per variable) as described in Peduzzi et al. (1996). The EPV value was compared with the threshold value 10 (Peduzzi et al. 1996). The classification performance of the fitted models was assessed by calculating the area under the relative operating characteristic curve (AUC) and its associated 95% confidence intervals with the method described in Robin et al. (2011). The resulting AUC was compared with the threshold value 0.5 Robin et al. (2011).

Based on the outcomes of the binary logistic regressions, pointing out that hail wound perimeter was the best predictor of the probability of infection by *C. parasitica* (see results), an appraisal on the association between hailstones size, hail wound perimeter and hence the risk of infection was conducted through a geometrical-based approach, hereafter referred to as geometrical approximation of hail wound model (GAHW). GAHW was aimed at modelling, in a three-dimension space, the shape and size of the wound created by the impact of a hailstone striking a shoot orthogonally to its growth axis, accounting for increasing contact forces. GAHW was designed and parametrized in order to build a graph relating the hail wound perimeter Wp (mm) as a function of the hailstone radius (R in mm), of the shoot radius (r in mm) and of the intensity of the impact. GAHW was designed by modelling the hailstone as a sphere of radius R (Field et al. 2010; Punge and Kunz 2016; Sun et al. 2015) and the shoot as a cylinder of radius r (Lione et al. 2016; Ross et al. 1998). Sphere and cylinder were located within a Cartesian space with orthogonal axes x, y and z and center O (0, 0, 0) (Lione et al. 2016) so that the cylinder axis (i.e. growth axis of the shoot in proximity of the wound) coincided with z, and the sphere was centered on the point C (x_c, 0, 0). Hence, the following set of equations was used to model the shoot (Equation 4) and the hailstone (Equation 5) in the space:

$$x^2 + y^2 = r \qquad \text{(Equation 4)}$$

$(\mathrm{x}-\mathrm{x}_c)^2+\mathrm{y}^2+\mathrm{z}^2=\mathrm{R}^2$ (Equation 5)

In this system, the hailstone falls along the direction of the x axis towards O striking the shoot with a first contact on point (r, 0, 0). Based on the equations reported in Puttock and Thwaite (1969) that describe the external orthogonal contact of a sphere and a cylinder driven by opposite forces, the shape of a hail-induced wound on a shoot was modelled as the geometric intersection between the sphere and the cylinder. This geometric intersection was assumed as a proxy of the wound resulting from the physical indentation α (Puttock and Thwaite 1969) or δ_{max} (Sun et al. 2015) created by the hailstone impact on the shoot. Hence, the wound perimeter was defined by the set of points belonging to both the sphere and the cylinder once the intersection had occurred. GAHW was run by setting as input variables the values of R, r and x_c. The first variable can be set by the user based on the observed or expected hailstorm intensity (i.e. hailstorm average radius), the second can be set based on the size of the branches, while the third varies during the fall of the hailstone, attaining its minimum value when the wound is created and hence the maximum indentation is reached. As the physical properties of chestnut shoots to parametrize the equations reported in Puttock and Thwaite (1969) and Sun et al. (2015) were not available in the literature, α (and hence x_c) could not be set directly. Therefore, since the indentation is directly proportional (i.e. ∝) to the radius of the hailstone [$\alpha \propto \|\vec{\mathrm{P}}\|$ see Puttock and Thwaite (1969), where $\|\vec{\mathrm{P}}\| = \|\vec{F_c}\| \propto v \propto D_{hail}$ see equations 1, 18, and A2 in Sun et al. (2015)] and it can be expressed either in the same unit of measurement of R, or as fraction of R (Puttock and Thwaite, 1969), hailstone impact on the shoot was assumed to reach the maximum depth of $\frac{\mathrm{R}}{\mathrm{n}}$. R and n values were consequently selected based on i) the visual observation aimed at appraising the approximate depth of hail wounds present on the sampled shoots, and ii) the expected range of hailstone radius according to the reconstruction of recent hailstorm events (see results) and to the climatologies available for the North West of Italy (Baldi et al. 2014; Punge and Kunz 2016). The range of r values was set according to the sizes of chestnut shoots comparable with those sampled in this study. Once the values of R, r and n had been fixed for each hailstone impact, the coordinates x_{Wp}, y_{Wp} and z_{Wp} of points lying on the spatial curve at the intersection of the sphere and the cylinder were calculated using Equation 6 by setting discrete steps of 10^{-5} radians for θ:

$$\begin{cases} x_c = \frac{n-1}{n}R + r \\ b = \frac{R^2 - r^2 - x_c^2}{2x_c} \\ \theta_0 = \arccos\left(-\frac{b}{r}\right) \\ -\theta_0 < \theta < \theta_0 \\ x_{Wp} = r \cdot \cos\theta \\ y_{Wp} = r \cdot \sin\theta \\ z_{Wp} = \pm\sqrt{2x_c(b + r \cdot \cos\theta)} \end{cases} \quad \text{(Equation 6)}$$

The calculation of the perimeter of each simulated hail wound on the shoot was approximated by summing the Euclidean distances between all consecutive points lying on the curve at the intersection of the sphere and the cylinder (Dobrow 2016). GAHW was run on all possible combinations of three representative values of n and r, plotting for any combination the graph displaying the relation between Wp and R.

The risk of blight insurgence at shoot level was obtained by integrating the GAHW model with the binary logistic regression modelling the probability of infection (%) by *C. parasitica* as a function of the hail wound perimeter.

For all analyses the 95% Bias Corrected and accelerated confidence interval was calculated for the averages based on 10^4 bootstrap resamplings (Carpenter and Bithell 2000; Carsey and Harden 2014; DiCiccio and Efron 1996). For proportions (e.g. incidences), exact 95% confidence intervals were calculated as reported in Blaker (2000). Both types of confidence intervals are abbreviated with the acronym CI throughout the text. Statistical analyses and modelling were conducted with R version 3.6.0 (R Core Team 2018) and with the associated packages bootstrap (Efron and Tibshirani 1994), HH (Heiberger and Holland 2015), MuMIn (Barton 2019), partykit (Hothorn and Zeileis 2015), pROC (Robin et al. 2011), rgl (Adler and Murdoch 2019) and strucchange (Zeileis et al. 2002). The significance threshold was set to 0.05 for all tests.

RESULTS

Assessing factors associated with dieback of chestnut trees

Results from the conditional inference tree models pointed out that the average score of crown transparency was significantly higher within the dieback area (53.7%, 50.4-57.0% CI) than outside (20.1%, 17.1-23.1% CI) ($P < 0.001$) (Table 2). The ECDFs of crown transparency scores assessed within and outside the dieback areas are shown in Supplementary file S2. Based on the outcomes of conditional inference tree models, the incidence of symptoms of chestnut blight was significantly higher within (92.0%, 83.7-96.5% CI) than outside (60.0%, 44.5-73.8% CI) the dieback area ($P < 0.001$) (Table 2). Conversely, the frequency of trees positive to the presence of *D. kuriphilus* galls was comparable and not significantly different ($P = 0.824$) within (88.0%, 79.0-93.8% CI) and outside the dieback area (84.4%, 71.4-92.9% CI), since the corresponding conditional inference tree models did not display any significant split. Hail-induced wounds on branches and shoots were present on 100% (95.2-100% CI) of the inspected trees in the dieback area, while they were significantly less prevalent (20.0%, 10.5-34.1% CI) outside the dieback area ($P < 0.001$), as confirmed by the presence of two terminal nodes in the corresponding conditional inference tree model (Table 2). In summary, for all the above mentioned comparisons, splits of conditional inference tree models occurred only in the comparison between sites within and outside the dieback area (Table 2). Hence, the levels of infestation of the Asian gall wasp were similar within and outside the dieback area, whereas the incidence of chestnut blight was significantly higher, in association with average levels of crown transparency exceeding 50%, in the dieback area compared to the surrounding stands.

Pathogen isolation and characterization of the hypovirulence

Isolations allowed to confirm the association between the presence of symptoms of blight on hail-induced wounds and presence of *C. parasitica*. In fact, 124 isolates of *C. parasitica* were obtained from symptomatic branches and shoots (isolation frequency 97.6%). No isolates of the pathogen were obtained from asymptomatic samples (isolation frequency 0%). Of the 124 isolates, 38 (30.6%) had a virulent, orange culture morphology, whereas 84 (67.7%) were characterized as intermediate. Only two isolates (1.6%) had a hypovirulent, white culture morphology. The TaqMan®-based real-time PCR assay confirmed the occurrence of Cryphonectria hypovirus-1 (CHV-1) in the white isolates as well as in six additional isolates classified as intermediate. All remaining isolates (93.5%) were virus-free.

Assessment of the relation between infection by *C. parasitica* and the number or size of hail wounds

The 359 branches and shoots collected during the second sampling had an average diameter of 9.9 mm (9.4-10.3 mm CI) and a mean length of 144.5 cm (137.7-151.9 cm CI). On them, a total of 12228 hail wounds and 1961 galls of *D. kuriphilus* were detected, corresponding to an average of 0.25 hail wounds/cm (0.23-0.26 hail wounds/cm CI) and 0.036 galls/cm (0.033-0.041 galls/cm CI). On average, a single hail wound measured 7.50 mm (7.40-7.61 mm CI) in length and 2.70 mm (2.66-2.73 mm CI) in width, with a perimeter of 17.1 mm (16.9-17.3 mm CI), a surface of 21.6 mm^2 (21.0-22.3 mm^2 CI) and an eccentricity of 0.846 (0.842-0.850 CI). On the whole, hail wounds associated with blight symptoms were 3285 out of 12228 (26.9%). However, they accounted for 50.4% of the total number of hail wounds observed on the symptomatic branches and shoots, while no hail wounds were associated with blight in asymptomatic branches.

By contrasting the 212 symptomatic and 147 asymptomatic branches and shoots, a significant split of the conditional inference tree model showed that the first displayed an average number of hail wounds significantly lower than the latter (30.7 *vs.* 38.9, $P = 2.701 \cdot 10^{-3}$). Nonetheless, in the 212 symptomatic branches and shoots the averages values of the shape and dimension variables of hail wounds L, W, S, p, and e were higher than those from the 147 asymptomatic samples, although not always significantly depending on the number of terminal nodes of the corresponding conditional inference tree models (Table 3). Galls abundance was significantly lower in asymptomatic than in symptomatic branches and shoots (4.34 *vs.* 7.08, $P < 0.001$), separated in two terminal nodes of the related conditional inference tree model. The ECDFs fitted to the number, average L, W, S, p, and e of hail wounds and to the number of galls of 212 symptomatic and 147 asymptomatic branches and shoots are shown in Supplementary file S3 along with the related histograms.

Results obtained for hail wounds and galls abundance were confirmed by their corresponding counterparts related to the length of the branches and shoots HWCL and GCL, respectively (Table 3).

Comparisons of size variables showed that on average hail wounds associated with blight symptoms were significantly ($P < 0.05$) larger than the others, with the associated conditional inference tree models displaying two terminal nodes (Table 4). On the total sample of branches and shoots, hail wounds colonized by *C. parasitica* significantly ($P < 0.05$) exceeded the dimensions of the other wounds by 24.7% for the length L, 15.8% for the width W, 45.8% for the surface S, and 23.6% for the perimeter p, as shown by the presence of significant splits in the related conditional inference trees (Table 4). Similar results were obtained from the splits displayed by the models fitted on the subset of symptomatic branches and shoots, where hail wounds associated with the pathogen significantly ($P < 0.05$) exceeded the wounds not associated with blight by 25.8% for L, 11.1% for W, 39.3% for S, and 23.6% for p. Moreover, the shape variable expressing the eccentricity of the hail wounds was significantly ($P < 0.05$) different in both cases, showing that wounds infected by *C. parasitica* were more stretched than those not colonized by the pathogen, since their e value was higher in both comparisons performed (Table 4).

Modelling the risk of infection of *C. parasitica* based on hail wounds shape and size

The binary logistic regression models pointed out that all tested shape and dimensions predictors were positively ($\beta > 0$) and significantly ($P < 0.05$) associated with the probability of infection by *C. parasitica* through hail induced wounds (Table 5). For all models the likelihood ratio test confirmed that increasing length, width, perimeter, surface and eccentricity of the hail wound significantly ($P < 0.05$) increase the risk of blight insurgence, although some of the above predictors outperformed the others in terms of model performances. In fact, based on the minimum AIC principle, M_p was the best scoring model, followed by M_L, M_S, M_W and M_e (Table 5). Based on AICw values, M_p is the most adequate model to describe the risk of infection with a probability of 97.9%, while M_L attained a probability of 2.1% and the others did not exceed the threshold of 0% (Table 5). M_p sigmoid curve (Fig. 3) shows that wounds perimeter below the threshold of 66 mm are associated with a probability of infection lower than 50% (46-53% CI), while for those with a perimeter of 107 mm the risk increases up to a probability of 70% (64-75% CI), which further grows over 90% (86-94% CI) for perimeters exceeding the 172 mm. M_p curve is shown in logit scale in Supplementary file S4. Most of the perimeter values used for fitting model M_p ranged from 0 to 100 mm, while values over 100 mm represented less than 1% of the sample size (Fig. 3). The EPV index attained the value of 3285, which was largely over the threshold of 10. The AUC value of model M_p was 0.59 with an associated 95% confidence interval ranging from 0.58 to 0.60, hence significantly over the 0.5 threshold.

Reconstruction of the hailstorm events

The interviews of the local farmers, forest owners and technicians of agricultural entrepreneurs association revealed that at least three intense hailstorms impacted the dieback area during 2012, the first occurring on May 10th, the second on June 18th and the third on August 22nd. Based on the descriptions provided during the interviews, hailstones size was “larger than a hazelnut” or “as big as a walnut”, hence it was estimated that the average diameter of the hail could be in the range 1-3 cm according to the indications reported in Baldi et al. (2014) and in the ANELFA scale (Dessens et al. 2007). Based on reports of hailstorm damage addressed to the municipality of Peveragno (S. Marchisio, COLDIRETTI Cuneo, *personal communication*) the event of June was probably the most intense, with a diameter of hailstones of about 3 cm, as confirmed by local newspapers (Prieri 2012) and by the ANELFA scale (Dessens et al. 2007). For the year 2011, at least one intense event was reported by half of the interviewed people, although there was no agreement on the exact timeframe, which was set approximately in the first half of November. Based on information reported by local online newspapers, the event could have taken place in association with the heavy rains and floods of November 6th (Cariddi 2011). The queries performed on the European Severe Weather Database and on the the Storm Report Meteonetwork Dataset did not provide any hailstorm record for the study area, while the the European Climate Assessment & Dataset and the Meteorologic and Idrologic Database of the Regional Agency for Environmental Protection (ARPA) of Piedmont did not contain specific information related to hailstorm occurrence.

Appraisal of the association between hailstones size, wound dimensions and risk of infection

The application of the GAHW approach resulted in a R algorithm (Supplementary file S5) allowing for the three-dimensional modelling of the shape of hail wounds on branches (Fig. 4). The virtual rendering of the hail wounds shape was visually consistent with the observations carried out on the chestnut samples (Fig. 5) and was in agreement with the elliptical approximation of the wound contour proposed in Costello (2014) and Schubert (1991).

R, r and n parameters that served as input for the GAHW model ranged between 1 and 25 mm for the hailstone radius R, between 5 and 20 mm for the shoot radius r, and between 3 and 10 for hailstorm severity parameter n. Within the above intervals, a discrete step of 1 mm was set for R, while the representative values 5, 10 and 20 mm were selected for r and 3, 5, and 10 for n. The outcome of the GAHW model (Fig. 6) showed that the hail wound perimeter increased linearly from values close to 1-5 mm up to values close to 100 mm with increasing hailstone radius from 1 to 25 mm. The rate of linear increase of the hail wound perimeter (i.e. slope of the linear plot) grows with decreasing values of n, and with increasing values of r (Fig. 6). The risk of blight insurgence at shoot level can be obtained by integrating the GAHW model (Fig. 6) with the binary logistic regression modelling the probability of infection (%) by *C. parasitica* as a function of the hail wound perimeter (Fig. 3). For instance, the GAHW model shows that during a severe hailstorm (n = 3), an increase of the hailstone radius from 5 to 25 mm increases the expected hail wound perimeter from 19 to 86 mm on a chestnut shoot of 5 mm radius. In this situation, the corresponding risk of infection by *C. parasitica* resulting from the binary logistic regression model increases from 27% to 60%.

DISCUSSION

By combining the outcomes of surveys conducted within and outside the dieback area with laboratory analyses and mathematical modelling, we were able to link the observed dieback of chestnut orchards and coppices to an outbreak of blight in turn triggered by hailstorm events. Furthermore, we identified the size of hail wounds as an important factor increasing the risk of *C. parasitica* infection and we observed that both virus-free and virus-infected strains of the pathogen may gain access into the host tissues through hail wounds.

The dieback appeared in the same area where both the Asian gall wasp (Avtzis et al. 2019) and chestnut nut rots caused by *G. castaneae* (Lione et al. 2019; Visentin et al. 2012) were first reported in Europe at the beginning of the century. However, despite *G. castaneae* has been reported in Switzerland to cause cankers similar to those of *C. parasitica* (Pasche et al. 2016), our isolations from symptomatic tissues would exclude any role of *G. castaneae* on the onset of symptoms, which instead appear to be caused exclusively by *C. parasitica*. The latter was never isolated from asymptomatic samples, although occasionally the fungus was reported to live endophytically in shoots and in the bark of *C. sativa* (Bissegger and Sieger 1994; Chandelier et al. 2019).

To determine the role of the different factors on the onset of the dieback, we combined the assessment of crown transparency with phytosanitary surveys and analyses, with an approach similar to that employed for studying diebacks of other tree species, including pines and oaks (Giordano and Gonthier 2011; Giordano et al. 2009; Gonthier et al. 2010; Jung et al. 2000; Lione et al. 2012). Infestations of the Asian gall wasp and chestnut blight appeared widespread, both within and outside the dieback area. However, while the levels of infestation of the Asian gall wasp were similar within and outside the dieback area, the incidence of chestnut blight was significantly higher, in association with average levels of crown transparency exceeding 50%, in the dieback area compared to the surrounding stands. Interestingly, it was recently shown that *C. parasitica* may infect abandoned galls of the Asian gall wasp (Meyer et al. 2015), thereby providing a clue supporting the hypothesis that chestnut blight could be the prevailing factor triggering the dieback. In addition, hail-induced wounds on small branches and shoots were observed with 100% incidence only in the dieback area suggesting either that the dieback was associated with the injuries caused by the hailstorms themselves or that those injuries may have facilitated infections of the chestnut blight pathogen. It is worth noting that the patchy distribution and the extension of the dieback area, clearly visible from satellite imagery, is fully consistent with the expected outcomes of hailstorm events striking a hilly region located within the complex orographic conditions of North West of Italy (Baldi et al. 2014; Punge and Kunz 2016). On the contrary, the above spatial distribution of the decline is hardly compatible with the dynamics of the infestation and spread of *D. kuriphilus* (Lione et al. 2016; Paparella et al. 2016). The major role played by hailstorms in the dieback is supported by the reconstruction of the hailstorm events. Data about hailstorms at the local, regional, national and European scale are notoriously difficult to obtain because of the lack of appropriate observation systems, and hence they are often and inevitably gathered based on reports from local people (Baldi et al. 2014; Mohr et al. 2015; Punge and Kunz 2016). These reports can be collected through interviews, by screening newspapers, or by querying databases from official institutions or datasets built thanks to the contribution of citizens and volunteers (Associazione ONLUS MeteoNetwork 2019; Baldi et al. 2014; Dotzek et al. 2009; Punge and Kunz 2016). Consulting as many relevant sources as possible is the key to gather reliable information from such citizen science-based approaches as we attempted to do in this study. In fact, as an example, the simple consultation of databases hinging on reports would not have been sufficient to detect the events we reconstructed from newspapers and interviews, simply because such events were not uploaded in the database systems, or because the system is still not adequate to distinguish hailstorms from other precipitations. In addition, as remarked by Dotzek et al. (2009), the frequency of reports feeding databases does not only depend on the frequency of events themselves, but also on the likelihood that events are reported by citizens and volunteers, thus highlighting once more the need of integrating the available multisource information. In this regard, the mosaic of information we gathered pointed out that at least four severe hailstorms had occurred in the dieback area. Remarkably, such hailstorms occurred during a timeframe consistent with the lifetime of the branches and shoots sampled in this study and with the onset of the blight symptoms observed. It is worth noting that event-based and non-systematic reports (Punge and Kunz 2016) might be biased in reconstructing only major hailstorm events and omitting the others. However, this potential bias is unlikely to affect our results, since the 12228 hail wounds we analyzed were created by all the hailstorms that occurred in the area, and not only by those hailstorms that our reconstruction could confirm. In fact, the ECDFs of the length and width measured for each of the above wounds display the presence of any wound size. Consistently, the histograms of the hail wound perimeters used to model the probability of infection by *C. parasitica* point out that all wound sizes are represented, despite they are unbalanced towards medium and small-size perimeters under 100 mm. This unbalance might influence the perfomance of the binary logistic regression modelling the risk of infection, as if it was used for extrapolation. However, this potential source of error is unlikely to affect significantly the reliability of our results, as confirmed by the analyses assessing the model perfomances and accounting for model uncertainty as well as for sample size adequacy (Grueber et al. 2011; Heiberger and Holland 2015; Hosmer and Lemeshow 1989; Peduzzi et al. 1996; Robin et al. 2011; Wagenmakers and Farrell 2004). In addition, the GAHW risk model we proposed (see below) accounts consistently not only for large-size hailstones related to the most severe hailstorms (Dessens et al. 2007), but also for all the others, including small-size hailstones. The empirical observation that the frequency and intensity of hailstorms have recently increased across the study area is confirmed by the available climatologies, pointing out that North West of Italy is at high risk of severe hail strikes, particularly in hill and mountainous areas (Baldi et al. 2014; Punge and Kunz 2016). Although we cannot exclude a direct role of hailstorms on the onset of the dieback, based on our isolation attempts we did find evidence that hail-induced wounds served as infection courts for *C. parasitica*. In addition, the incidence of chestnut blight was significantly higher in the dieback area compared to the surrounding stands further supporting the hypothesis that infections leading to dieback may have been driven by hailstorms. Hence, by attempting to classify the role of the different factors in the framework of the forest decline concepts (Manion 1991), we propose hailstorms as an inciting factor and the chestnut blight as a contributing factor of chestnut decline. In fact, as we showed in this study, hailstorms foster the infection of the chestnut blight pathogen and, possibly, behave as relevant stressors for the tree. It should be noted that some of the hailstorms putatively associated with the observed dieback occurred during the spring or in association with rainfalls in the fall providing a clue to explain massive infections because masses of infectious *C. parasitica* pycnospores are generally present in those periods of the year or in association with precipitations (Guérin et al. 1999; Robin and Heiniger 2001). In addition to wounds on small branches and shoots, hailstorms caused holes and ripping on leaves which, in turn, predictably affected the process of evapotranspiration, which is particularly active in the spring and summer. Such a phenomenon may have had effects similar to those of drought conditions, which have also been previously suggested to enhance the occurrence of *C. parasitica* infections (Prospero and Rigling 2013). Finally, we cannot exclude that the infestation of the Asian gall wasp, as well as other factors, including climate change and the age of trees, may have played the role of predisposing factors. In fact, the severity of the dieback may have been different in the absence of the infestation. However, our data does not allow testing of this hypothesis.

Data obtained from laboratory analyses allowed to point to a clear role played by the size of hail wounds rather than by their abundance as a factor triggering infections. It is worth noting that, despite the operational constraints resulting in a slightly unbalanced sample of symptomatic and asymptomatic branches and shoots, the conditional inference tree models we used are notoriously robust and reliable since they are based on algorithms hinging on unbiased recursive partitioning and conditional inference (Hothorn and

Zeileis 2015; Hothorn et al. 2006). Indeed, infected shoots displayed a lower average number of hail wounds than uninfected shoots, yet the hail wounds associated with blight were significantly larger in size and more stretched in shape. This result is consistent with the infection dynamics and the epidemiology of *C. parasitica*, which is acknowledged as a pathogen whose infections are prompted by the presence of wounds at the stem, branch and shoot levels (Prospero and Rigling 2013). Not surprisingly, the potential role of hailstorms in enhancing the occurrence of *C. parasitica* infections was previously hypothesized (EFSA PLH Panel 2014; Turchetti et al*.* 2010). Nonetheless, this study reports the first experimentally-based evidence providing quantitative information about the association between hail wounds, the infection process and the development of chestnut blight. Since infections by *C. parasitica* occur mainly through inoculum passively dispersed by air or water and deposited on the surface of injuries exposing the cambial tissue (Prospero and Rigling 2013), the infection can be deemed as a stochastic process (see Lione and Gonthier 2016 and references within). Different stochastic models have been proposed so far to describe how spores and other particles endowed with comparable physical properties can spread and land, including random walks (Bicout and Sache 2003; Stockmarr 2002), the Ornstein–Uhlenbeck process (Dobrow 2016), the Lagrangian stochastic particle dispersion model (Kuparinen et al. 2007) and the homogeneous Poisson process (Illian et al. 2008). Since the probability that a spore will land on a specific surface depends on the surface dimension (Stockmarr 2002), an increased probability of spore deposition on the injury produced by hail might be reasonably expected with increasing dimensions of the wound surface. However, extensive Monte Carlo simulations studies (see for example Carsey and Harden 2014; Dobrow 2016; Lione and Gonthier 2016) based on the stochastic models listed above should be conducted to support this hypothesis.

Although all predictors related to the size of the hail wounds (length, width, perimeter and surface) were significantly associated with the probability of infection by *C. parasitica*, the perimeter was the most adequate for risk assessment. This result may be interpretable by considering that *C. parasitica* is a fungal pathogen infecting mainly the thin cambium layer under the bark, rather than the inner plant tissues. Hence, once the wound has been created by the impact of a hailstone, the cambial tissue available for infection is likely to be located towards the perimeter of the injuries, rather than on the central portion. Nonetheless, no lines of evidence are currently available to support this hypothesis.

Based on the logistic models proposed in this study, it is now possible appraising the risk of infection by *C. parasitica* through the measurement of the length and width of the hail wound. While this approach can be useful for in-field monitoring, risk assessment and modelling, it does not suffice for the appraisal of which hailstorm intensity could boost the onset or resurgence of the chestnut blight. For this purpose, we attempted to appraise the association between hailstones size, hail wound perimeter and the risk of infection through the newly designed geometrical-based model GAHW. The GAHW model relies on the assumption that the damage caused by hail strikes on chestnut is directly correlated with the size of hailstones. Although other variables may influence the magnitude of the damage caused by hailstorms, such as hail density, wind speed and direction (Changnon et al. 2009), hail size is by far the main indicator of hailstorm-related damages (Punge et al. 2014). Not surprisingly, many studies were focused on the importance of hailstone size (Baldi et al. 2014). For instance, agricultural losses are expected to occur when the hailstone size increases over the threshold of 5 mm (Punge and Kunz 2016). Damages to wheat, corn and soybean crops are more likely if the diameter of hailstone is over the cut-off value of 6.35 mm (Changnon 1971). In addition, inventories referring to economic losses of the major hailstorm events observed in Europe from 1788 to 2014 relate to the diameter of the hailstones (Punge and Kunz 2016). For the above reasons, hailstone size along with its kinetic energy stand among the main indicators of hailstorm severity included in damage models (Punge et al. 2014; Vinet 2001; Walsh et al. 2016), as well as in hail intensity/damage scales such as ANELFA (Dessens et al. 2007). It is worth noting that damage is related to the logarithm of the hail kinetic energy, which is proportional to the 4^{th} power of the hailstone diameter (Baldi et al. 2014). Hence, the assumption that hailstones size and hail wound perimeter are correlated seems to be supported by the lines of evidence listed above, while the relation linking the perimeter and the risk of infection by *C. parasitica* was unraveled by our binary logistic regression model. The GAHW model provided a three-dimensional rendering of the hail wound that was consistent both with the observations of the hail injuries observed on the sampled shoots and with the information reported in the literature (Costello 2014; Schubert 1991). One of the main constraints of this model is the geometrical rather than the physical approach. Nonetheless, the state of the art about the static and dynamic physical properties of young branches and shoots of chestnut is unlikely to allow a reliable parametrization of a physical-based model. In fact, even under the simplification introduced in GAHW, the minimal information required to calculate the equations proposed by Puttock and Thwaite (1969) and Sun et al. (2015) is not available, hampering the precise assessment of the forces involved and the depth of the hailstone penetration during the impact. The same issue does not allow a reliable modelling of non-orthogonal impacts. Nonetheless, there is a general agreement about the direct proportionality linking hailstone radius, velocity, impact force and depth of penetration in the stricken target (Baldi et al. 2014; Field et al. 2010; Punge and Kunz 2016; Puttock and Thwaite 1969; Sun et al. 2015). Hence, the choice of expressing the hailstorm severity through the GAHW parameter (n), relating the indentation of the hail wound to a fraction of the hailstone radius, holds reasonable. It is worth noting that this choice allows to account for other factors that, in addition to hailstone size, could influence the severity of the impact, with emphasis on wind. Indeed, windy hailstorms may increase the hailstones kinetic energy and consequently produce damages more detrimental than those resulting from a hailstorm with the same hailstones size, but in the absence of wind (Baldi et al. 2014; Towery et al. 1976). Consistently, the GAHW model shows that a reduction in the value of n accounting for an increased intensity of the hailstone impact generates larger hail wounds for any given hailstone size. The estimates of the wound depth through n values set from 3 to 10 seem consistent with the dimensions of hailstones reported by local people in the study area, with the average dimensions of hailstones in Italy (Baldi et al. 2014) and with the depth of the hail wounds observed in the sampled branches and shoots of chestnut. Interestingly, while the field data showed a range of hail wound perimeters up to approximately 300 mm, GAHW model produced as output a maximum of approximately 100 mm. Since hailstones with a radius over 20 mm occur only in 3.5% of cases (Punge and Kunz 2016) and considering that the difference between 300 mm and 100 mm is too large to only depend upon potential errors in input parameters, the most likely explanation relies in the type of impact of hailstone on shoot. In fact, GAHW is based on the assumption of orthogonality between the hailstone trajectory and the growth axis of the shoot, a condition that might not be met in the field especially during windy and turbulent hailstorms. Hence, it seems reasonable inferring that perimeters over 100 mm are caused by non-orthogonal impacts. The corresponding hail wounds are consequently expected to be more severe based on the increased kinetic energy of the hailstones (Baldi et al. 2014) and potentially more stretched. This hypothesis is confirmed by the fact that hail wounds with a higher eccentricity were associated with a significantly higher risk of infection by *C. parasitica*. Nonetheless, the bark thickness could also play a role by potentially affecting the shape and dimensions of hail wounds, although this factor would require a different experimental design to be accounted for in a risk model. GAHW is the first model that could be used to estimate the risk of infection by *C. parasitica* based on hailstone size. However, further studies of contact physics, wood technology and hailstorm meteorology are needed to improve the risk assessment of chestnut blight insurgence.

On the whole, 6.5% of *C. parasitica* isolates analyzed in this study through the newly developed TaqMan®-based real-time PCR assay were infected by Cryphonectria hypovirus-1 (CHV-1). Unfortunately, no specific studies have been carried out to investigate the prevalence of hypovirulence at the population level in the North West of Italy, which may have been helpful for comparative purposes. In a recent study conducted in different chestnut stands in Europe, the percentage of isolates bearing CHV-1 ranged from 6.9% to 61.5%, depending on site (Ježić et al. 2019). Thus, hypovirulence in our study sites seems to be low in comparison with the range reported by Ježić et al. (2019), which may provide a further clue to interpret the severity of the dieback. Whether the low prevalence of hypovirulence in our samples may be a trait linked either to the pathogen populations in the area, including to the diversity of their vegetative compatibility groups (VCGs), or to the selection for virulent strains of the pathogen during infection through hail wounds remains an open question deserving further studies. Nevertheless, the detection of CHV-1 in our fungal samples clearly indicates that infections incited by hail wounds may involve both virus-free (virulent) and virus-infected (hypovirulent) strains of the pathogen.

Locally distributed diebacks of chestnut orchards and coppices were also reported elsewhere in Piedmont, North West of Italy. Although no detailed investigations were conducted to unravel the causes of those diebacks, field surveys confirmed that those additional dieback areas had been challenged by both chestnut blight and hailstorms, supporting a role of these two factors in the occurrence of diebacks. It is worth noting that Piedmont owns some of the highest records of hailstorm events in Italy (Baldi et al. 2014; Punge and Kunz 2016). Although forecasts are challenging in this sense, it seems likely that an increased frequency and severity of hailstorm events might be expected for the near future (Baldi et al. 2014; Punge and Kunz 2016) as a result of anthropogenic climate change leading to the alteration of large-scale circulation and weather patterns (Brimelow et al. 2017; Kunz et al. 2009).

In conclusion, in this study we provided a new and quantitative-based piece of information on the effects of hailstorms on the epidemiology of *C. parasitica* while exploring the factors involved in a sudden dieback of chestnut trees in Northern Italy. Since the frequency and possibly the intensity of hailstorms are on the rise and chestnut blight is highly prevalent in most of the natural range of chestnut, diebacks observed in the North West of Italy may become more and more common.

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TABLES

Table 1. Primers and TaqMan® probes used in this study.

Primers and TaqMan® probes name	Sequence
CPTub-RealF	5' CGCAACGGTCGCTACCTG 3'
CPTub-RealRev	5' TGCGCATCTGGTCCTCG 3'
CPTub-TaqMan® probe	5' [6FAM] GCTCTGCCATCTTCCGTGGTAAGGTCT[TAM]
CHV1-REalF	5' ACCTGGTTCGCCGAAGAAC 3'
CHV1-RealRev	5' GCAACCTCTAAGGCAACCAATT 3'
CHV1-TaqMan® probe	5' [6FAM] CAGACACGTTCTGGCCCGACTGG[TAM]

Table 2. Mean crown transparency (MCT) values and incidence of chestnut blight symptoms (ICBS), of *Dryocosmus kuriphilus* galls (IDK), and of hail-induced wounds (IHIW) in the study sites located within and outside the dieback area. Values are reported along with their related 95% confidence interval (CI). Different letters indicate that the associated values are significantly different (P < 0.05) since they are split in two different terminal nodes by the conditional inference tree model. Conversely, the same letter indicates values not significantly different (P > 0.05), that are included within the same node of the conditional inference tree model. Comparisons conducted with such models are reported columnwise in the table.

Study site	MCT	ICBS	IDK	IHIW	Study site location	MCT	ICBS	IDK	IHIW
1o	19.3 (14.3-25.3)b	73.3 (46.5-90.3)b	73.3 (46.5-90.3)a	13.3 (2.4-39.4)b	Outside the dieback area	20.1 (17.1-23.1)b	60.0 (44.5-73.8)b	84.4 (71.4-92.9)a	20.0 (10.5-34.1)b
2o	23.3 (18.0-28.3)b	46.7 (21.5-71.0)b	86.7 (60.6-97.6)a	20.0 (5.7-46.5)b					
3o	17.7 (13.3-22.0)b	60.0 (33.2-81.3)b	93.3 (69.8-99.7)a	26.7 (9.7-53.5)b					
1w	53.7 (47.7-58.3)a	100 (78.5-100)a	86.7 (60.6-97.6)a	100 (78.5-100)a	Within the dieback area	53.7 (50.4-57.0)a	92.0 (83.7-96.5)a	88.0 (79.0-93.8)a	100 (95.2-100)a
2w	50.3 (41.7-58.3)a	86.7 (60.6-97.6)a	86.7 (60.6-97.6)a	100 (78.5-100)a					
3w	56.7 (50.3-62.0)a	93.3 (69.8-99.7)a	93.3 (69.8-99.7)a	100 (78.5-100)a					
4w	59.3 (52.0-66.6)a	93.3 (69.8-99.7)a	86.7 (60.6-97.6)a	100 (78.5-100)a					
5w	48.7 (41.0-57.3)a	86.7 (60.6-97.6)a	86.7 (60.6-97.6)a	100 (78.5-100)a					

Table 3. Comparison of hail wounds number, shape and size variables, and of galls abundance between symptomatic and asymptomatic branches and shoots. Average values are reported along with their related 95% confidence interval (CI). Different letters indicate that the associated values are significantly different (*P* < 0.05) since they are split in two different terminal nodes by the conditional inference tree model. Conversely, the same letter indicates values not significantly different (P > 0.05), that are included within the same node of the conditional inference tree model. The variables significantly different are marked with asterisks. Comparisons are reported rowwise in the table along with the corresponding *P*-value. HWC: hail wounds count (number of wounds); HWCL: hail wounds count related to the length of the branches and shoots (number of wounds/cm); L: length of the hail wound (mm); W: width of the hail wound (mm); S: surface of the hail wound (mm^2); p: perimeter of the hail wound (mm); e: eccentricity of the hail wound.

Variable	Symptomatic branches and shoots	Asymptomatic branches and shoots	*P*-value
HWC*	30.7 (27.7-34.1) b	38.9 (34.8-43.4) a	$2.701 \cdot 10^{-3}$
HWCL*	0.21 (0.19-0.23) b	0.30 (0.28-0.33) a	< 0.001
L	7.89 (7.52-8.32) a	7.40 (7.07-7.77) a	$9.575 \cdot 10^{-2}$
W*	2.73 (2.60-2.86) a	2.53 (2.41-2.66) b	$4.213 \cdot 10^{-2}$
S*	22.8 (20.7-25.4) a	19.3 (17.7-21.0) b	$2.818 \cdot 10^{-2}$
p	17.9 (17.1-18.8) a	16.7 (16.1-17.5) a	$5.998 \cdot 10^{-2}$
e	0.863 (0.851-0.873) a	0.846 (0.828-0.861) a	$8.849 \cdot 10^{-2}$
GC*	4.34 (3.71-5.06) a	7.08 (5.89-8.57) b	< 0.001
GCL*	0.027 (0.023-0.031) a	0.050 (0.043-0.060) b	< 0.001

Table 4. Comparison of shape and size variables between hail wounds associated or not with blight symptoms. Comparisons are performed rowwise both on the whole sample of chestnut branches and shoots, and on the subset of the symptomatic branches and shoots. Average values are reported along with their related 95% confidence interval (CI). Different letters indicate that the associated values are significantly different ($P < 0.05$) since they are split in two different terminal nodes by the conditional inference tree model. The variables significantly different are marked with asterisks. L: length of the hail wound (mm); W: width of the hail wound (mm); S: surface of the hail wound (mm^2); p: perimeter of the hail wound (mm); e: eccentricity of the hail wound.

Variable	Hail wounds associated with blight	Hail wounds not associated with blight	*P*-value	Hail wounds associated with blight in symptomatic branches and shoots	Hail wounds not associated with blight in symptomatic branches and shoots	*P*-value
L*	8.78 (8.53-9.06) a	7.04 (6.93-7.15) b	< 0.001	8.78 (8.53-9.06) a	6.98 (6.80-7.17) b	< 0.001
W*	3.00 (2.93-3.08) a	2.59 (2.55-2.62) b	< 0.001	3.00 (2.93-3.08) a	2.70 (2.64-2.77) b	< 0.001
S*	28.0 (26.4-30.0) a	19.2 (18.6-19.9) b	< 0.001	28.0 (26.4-30.0) a	20.1 (19.0-21.5) b	< 0.001
p*	19.9 (19.3-20.4) a	16.1 (15.9-16.3) b	< 0.001	19.9 (19.3-20.4) a	16.1 (15.7-16.5) b	< 0.001
e*	0.871 (0.864-0.876) a	0.837 (0.832-0.842) b	< 0.001	0.871 (0.864-0.876) a	0.856 (0.849-0.862) b	< 0.001

Table 5. Binary logistic regressions modelling the probability (%) of infection by *Cryphonectria parasitica* as a function of the shape and dimension of the hail-induced wounds. Models M_L, M_W, M_p, M_S, and M_e include as single predictors the length (L, in mm), width (W, in mm), perimeter (p, in mm), surface (S, in mm^2) and eccentricity (e) of the hail wounds, while M_0 is the null model. For the estimates of the intercept (β_0) and the predictors' coefficients (β), the associated standard errors (SE) and Wald's test *P*-value (*P*) are reported. The Akaike information criterion (AIC), AIC weight (AIC_w) and the likelihood ratio test (LRT) *P*-value (*P*) are shown for each model. Asterisks next to the β or β_0 values indicate significant (*P* < 0.05) Wald tests outcomes, while asterisks next to the model label mark significant (*P* < 0.05) likelihood ratio tests.

Model	β_0	**SE(β_0)**	***P*(β_0)**	β	**SE(β)**	***P*(β)**	**AIC**	**AIC_W**	***P*(LRT)**
M_0	-1.001*	$2.04\ 10^{-2}$	< 0.001	-	-	-	14233.1	0	-
M_L^*	-1.336*	$3.32 \cdot 10^{-2}$	< 0.001	$4.295 \cdot 10^{-2}$*	$3.28 \cdot 10^{-3}$	< 0.001	14056.6	0.021	< 0.001
M_W^*	-1.303*	$3.56 \cdot 10^{-2}$	< 0.001	$1.085 \cdot 10^{-1}$*	$1.02 \cdot 10^{-2}$	< 0.001	14124.0	0	< 0.001
M_p^*	-1.368*	$3.48 \cdot 10^{-2}$	< 0.001	$2.070 \cdot 10^{-2}$*	$1.55 \cdot 10^{-3}$	< 0.001	14048.9	0.979	< 0.001
M_S^*	-1.130	$2.41 \cdot 10^{-2}$	< 0.001	$5.662 \cdot 10^{-3}$*	$5.50 \cdot 10^{-4}$	< 0.001	14119.0	0	< 0.001
M_e^*	-1.656	$9.32 \cdot 10^{-2}$	< 0.001	$7.655 \cdot 10^{-1}$*	$1.05 \cdot 10^{-1}$	< 0.001	14176.3	0	< 0.001

FIGURES

Fig. 1. Hills covered by chestnut orchards and coppices in the municipality of Peveragno, North West of Italy. Severe dieback of chestnut trees occurred following a patchy spatial pattern, with a rather sharp separation between areas showing dieback symptoms and the surrounding areas.

Fig. 2. Location of the study sites in the North West of Italy, municipality of Peveragno. Sites within the dieback area of chestnut (1w, 2w, 3w, 4w and 5w) are marked in red, while sites located outside the dieback area (1o, 2o, 3o) are highlighted in green. Satellite imagery (Google 2019) and shaded relief background (ESRI 2019) were accessed through QGIS (QGIS Development Team 2019) with QuickMapServices plugin version 0.19.11.1.

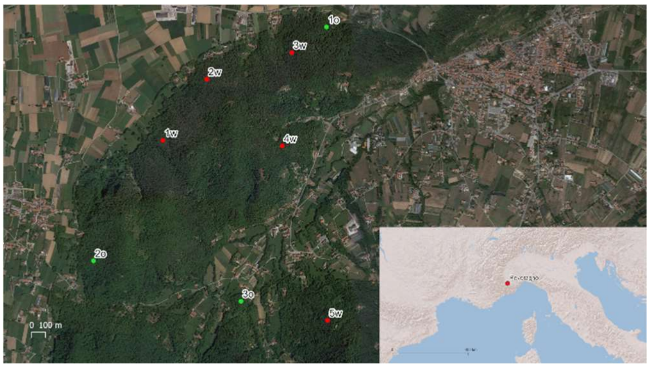
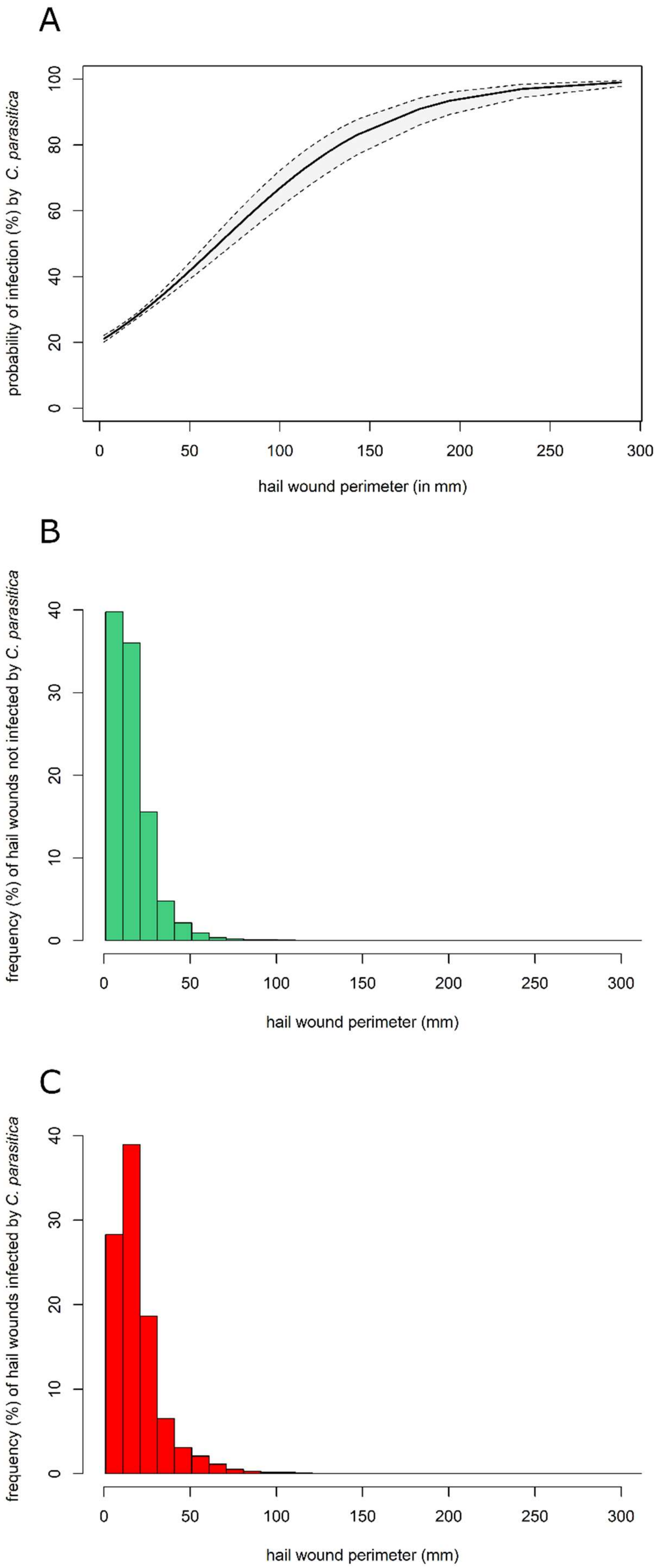


Fig. 3. Sigmoid curve of the binary logistic regression modelling the probability of infection (%) by *Cryphonectria parasitica* as a function of the hail wound perimeter (in mm) (panel A). The region comprised between the contours above and below the curve delimits the 95% confidence interval of the predicted probability (panel A). The frequency (%) of hail wounds used for the model fitting is shown as a function of the perimeter (in mm) in histograms of wounds not infected (panel B) and infected by *C. parasitica* (panel C).



A
probability of infection (%) by C. parasitica
0
20
40
60
80
100
0
50
100
150
200
250
300
hail wound perimeter (in mm)
B
frequency (%) of hail wounds not infected by C. parasitica
0
10
20
30
40
0
50
100
150
200
250
300
hail wound perimeter (mm)
C
frequency (%) of hail wounds infected by C. parasitica
0
10
20
30
40
0
50
100
150
200
250
300
hail wound perimeter (mm)

Fig. 4. Three-dimensional modelling of the shape of hail wounds on shoots based on the geometrical approximation of hail wound (GAHW) model. Panel A shows the impact of a hailstone (gray sphere) on the shoot surface (green cylinder) with the related indentation associated with the hail injury. Panel B displays the hail wound perimeter where red points mark the hail wound width (distance from E_1 to E_2), length (distance from E_3 to E_4) and the point of maximum depth reached by the wound (E_5).

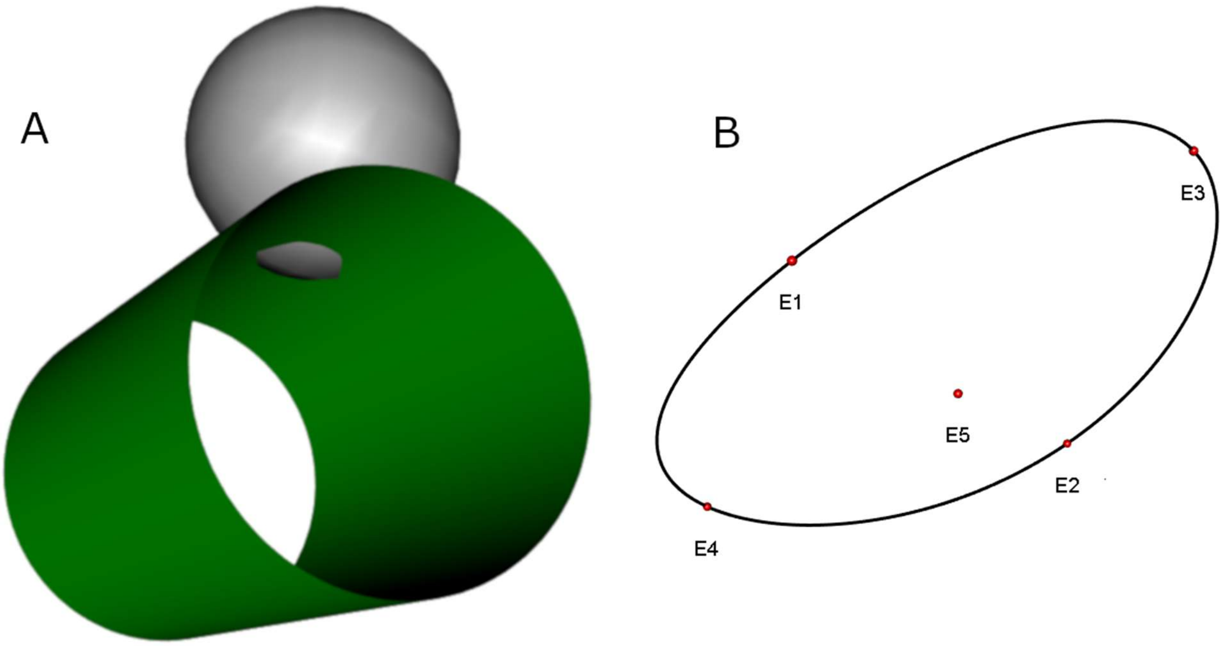


Fig. 5. Examples of hail-induced wounds detected on chestnut shoots sampled for this study. Panel A shows hail-induced wounds not associated with *Cryphonectria parasitica* infection. The Panel B displays hail-induced wounds associated with symptoms of blight; orange fruiting bodies of *C. parasitica* emerge from the bark.

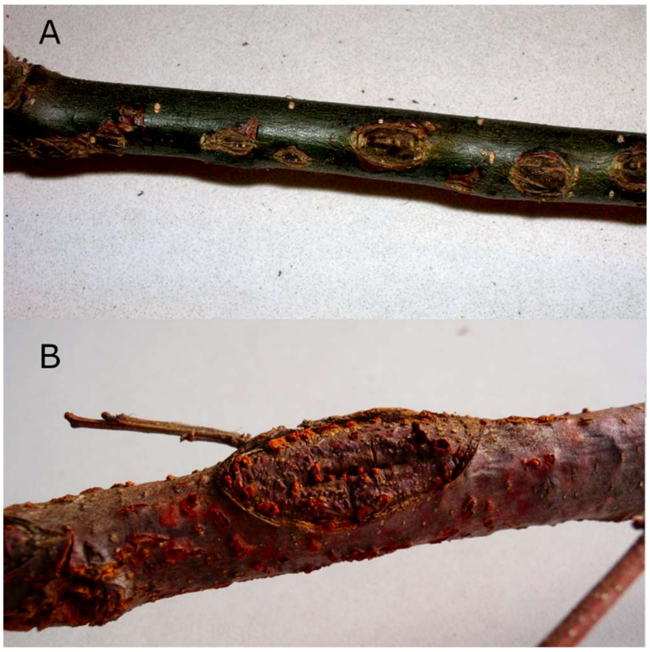


Fig. 6. Geometrical approximation of hail wound (GAHW) model relating the hail wound perimeter (mm) to the hailstone radius (mm) for increasing levels of hailstorm severity (from n = 10 to n = 3) and radius of the shoot (from r = 5 mm to r = 20 mm). Values of n are inversely proportional to hailstorm severity.