Dynamics of *Cryphonectria hypovirus* Infection in Chestnut Blight Cankers

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ABSTRACT

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Virulent strains of the chestnut blight fungus *Cryphonectria parasitica* cause lethal bark cankers on chestnut trees. Infection of *C. parasitica* with *Cryphonectria hypovirus 1* in Europe biologically controls this disease, leading to nonlethal and inactive cankers. Unexpectedly, virus-free *C. parasitica* strains have been isolated from inactive cankers. In this study, we compared the virulence of virus-infected and virus-free *C. parasitica* strains isolated from either inactive or active cankers on chestnut seedlings and sprouts. In the seedling experiment, we assessed canker growth and seedling mortality. In the sprout experiment, we also assessed canker growth and made fungal reisolations to determine virus infection

and immigration of foreign vegetative compatibility (vc) types over a period of 13 years in a coppice forest. Overall, the virulence of virus-free *C. parasitica* strains isolated from inactive versus active cankers did not differ. Significant differences were only attributed to virus infection. Virus infection and fungal strain composition in cankers changed over time. Foreign vc types immigrated into cankers and virus-free cankers became virus-infected within a few years. Most of the cankers were callused over time and became inactive. However, we observed that the virus did not always persist in these cankers. This study demonstrates that virus spread occurs effectively in European chestnut forests and that this biocontrol system is highly dynamic.

Additional keywords: *Castanea sativa*.

Chestnut blight is a serious tree disease in forests and orchards. Responsible for this disease is the ascomycete fungus *Cryphonectria parasitica* (3,24). The pathogen infects the bark and cambium of the tree through wounds and causes lethal bark cankers on susceptible chestnut (*Castanea* spp.). Active cankers usually enlarge rapidly and assume a sunken appearance due to the death of the cambium under the infected bark. The cankers may girdle the branch or stem and induce death to all plant parts distal to the infection, eventually killing the entire tree (24,26). *C. parasitica* is native to East Asia, where chestnut blight is only a minor problem because the local chestnut species (primarily *Castanea mollissima* and *C. crenata*) are resistant to the disease (19).

Cryphonectria parasitica was accidentally introduced to North America in ≈1900 and to Europe in ≈1930 (12,14). The American and European chestnut (*Castanea dentata* and *C. sativa*, respectively) had not coevolved with *C. parasitica* and, thus, were highly susceptible (1). After introduction, *C. parasitica* spread rapidly throughout the local chestnut populations. In a devastating epidemic, the disease destroyed the native chestnut forests in North America by killing most of the mature American chestnut trees (2).

In Europe, the epidemic took a similar course in the beginning. Surveys in natural populations of European chestnut revealed a general susceptibility to the disease, with little variation among trees (4). Nevertheless, a few years after introduction, superficial, sometimes callusing, cankers were observed that eventually stopped expansion and became inactive (14). These inactive

cankers assumed a dark appearance with quiescent margins and were colloquially designated as “healed” cankers. Responsible for the recovery of the cankers was an infection of *C. parasitica* with *Cryphonectria hypovirus 1* (CHV-1). CHV-1 is an unencapsidated RNA virus of the genus *Hypovirus* (9,16). The hypovirus (hereafter referred to simply as “virus”) does not kill *C. parasitica* but it reduces the pathogenic potential of the fungus, a phenomenon called hypovirulence (29). It further inhibits sexual reproduction, strongly attenuates growth and asexual sporulation, and reduces pigmentation of *C. parasitica* (19).

CHV-1 was presumably introduced together with its host, *C. parasitica*, to Europe and spread naturally throughout the *C. parasitica* populations by transmission from one fungal individual to another (7). In European regions where natural hypovirulence did not appear, the rapid dissemination of CHV-1 was further aided by active biocontrol efforts (15). To date, chestnut blight incidence in Europe is high but, due to hypovirulence, the disease is maintained at low severity in most regions (6,19,27).

The *Cryphonectria*–hypovirus pathosystem is a famous textbook example for a successful biocontrol system using mycoviruses (1,20). Prevalence of CHV-1 and induced hypovirulence in *C. parasitica* has most likely prevented a devastating chestnut blight epidemic and the destruction of the European chestnut forests to date. Therefore, in this study, we wanted to investigate the dynamics of this biocontrol system. As described above, inactive cankers are generally associated with virus infection. However, *C. parasitica* isolations revealed that not only virus-infected but also virus-free *C. parasitica* strains are frequently recovered from inactive cankers (5,8,23). This is unexpected and raises the question of whether inactive cankers can also be the result of virus-free *C. parasitica* strains with naturally low virulence. To address this question, we tested and compared the virulence of virus-free *C. parasitica* strains isolated from inactive cankers and the virulence of virus-free *C. parasitica* strains isolated from active cankers. An alternative hypothesis explaining

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the observation of virus-free *C. parasitica* strains in inactive cankers would be the lack of persistence of CHV-1 in these cankers over time. To explore the potential of this hypothesis, we assessed the presence of the virus in cankers over time by repeated *C. parasitica* isolations from the same cankers. Furthermore, we assessed the vegetative compatibility (vc) types of these *C. parasitica* isolates. *C. parasitica* vc types are determined by at least six bi-allelic genetic loci (10), which enabled us to detect potential immigrations of new fungal strains into the cankers.

MATERIALS AND METHODS

***C. parasitica* strains.** In this study, both virus-free and virus-infected *C. parasitica* strains were used. All strains were isolated in 1994 from either active or inactive chestnut blight cankers (Fig. 1; Table 1) in two naturally infected chestnut coppice forests in Ticino, Switzerland. Active cankers exhibit a red, actively growing border and are rapidly expanding. In contrast, inactive cankers are characterized by a dry, black surface and quiescent margins.

Seedling inoculation experiment. For this experiment, five different *C. parasitica* strains of each of four types were used: O-active, O-inactive, W-active, and W-inactive (Table 1). In addition, one virus-free (O-control) and one virus-infected (W-control) strain from our culture collection at WSL were used as controls. Each strain was grown on potato dextrose agar (PDA) Difco, Voight Global Distribution, Lawrence, MD) at 25°C in the dark for 5 days and then inoculated onto five 4-year-old chestnut seedlings. The seedlings were offspring of the same mother tree grown in a nursery. One circular wound was made with a cork

borer (5 mm in diameter) into the stem of each seedling and filled with two mycelia mats of the same strain obtained from the growing edge of the PDA culture. The wounds were then covered with transparent adhesive tape to prevent desiccation. For the duration of the experiment, all infected seedlings were kept in a nursery plot outdoors in Bellinzona (Ticino), southern Switzerland. All seedling inoculations were performed on 26 June 1995. Canker area and seedling mortality were used as measures for virulence of the strains. The canker area on each seedling was assessed 2 months (August 1995), 6 months (December 1995), and 13 months (July 1996) after inoculation by measuring two diameters of each lesion, one along the longitudinal and a second along the lateral axis of the stem. Because the inoculations killed a large proportion of seedlings during summer 1996, no further canker measurements were used for the analysis thereafter. We assessed the mortality of seedlings by recording the number of seedlings that had died by 27 September 1996.

Sprout inoculation experiment. In this experiment, three different *C. parasitica* strains of type O-active and three strains of type O-inactive were used. One virus-free (O-control) and one virus-infected (W-control) strain were used as controls. Inoculations of sprouts were performed as described for the seedling inoculation experiment. Five chestnut sprout clusters composed of at least eight individual sprouts were inoculated with each *C. parasitica* strain. Sprouts of the same cluster were growing from a single stump and were clones of the same tree. In the experiment, each sprout cluster was equivalent to one of five blocks and one replicate of each *C. parasitica* strain was used for one inoculation within each block. The sprouts were 11 years old and situated in a coppice forest in Lumino (Ticino), southern

Fig. 1. Active (left) and superficial, inactive (right) chestnut blight cankers.

Switzerland, where natural hypovirulence is present (5). Inoculations were performed on 26 June 1995. The canker areas produced by the strains were measured 6 months (November 1995), 15 months (September 1996), 30 months (November 1998), and 39 months (September 1998) after inoculation, as described above.

In addition, we visually assessed the morphological development of all cankers at five time points: March and November 1997, September 1998, November 1999, September 2000, August 2004, and September 2008. We distinguished between active, callusing, and callused cankers. Active cankers showed a red, actively growing border and were often sunken in appearance, exposing the wood of the stem. In callusing cankers, callus tissue started to wall off the lesion (Fig. 2). The wood of the stem was still exposed but the canker stopped expanding. In completely callused cankers, the lesions were finally totally walled off with newly formed bark tissue and the wood was no longer exposed. It is important to note that these morphological features of callusing and callused cankers are characteristic for chestnut blight lesions that had been initiated artificially by wounding the tree to the depth of the cambium. In the case of natural infection, inactive cankers are often superficial and characterized by a dry black surface, as described above (Fig. 1). In our assessment, we also recorded dead sprouts and recorded whether or not they had died due to *C. parasitica* infection. A girdling canker that had killed the sprout above the infection site indicated mortality caused by *C. parasitica*.

To determine the presence or absence of virus infection or to assess the invasion of foreign vc types into the chestnut blight canker, *C. parasitica* reisolations were performed at five different points in time: March and November 1997, September 2000, August 2004, and September 2008. At each time, three bark samples per canker were taken with a cork borer (5 mm in diameter) (i.e., one at the top, one in the middle, and one at the bottom of the canker). The cork borer was flame sterilized with

70% ethanol between cankers. Pure cultures of *C. parasitica* were obtained by first isolating the fungus from the surface-sterilized bark samples on water agar followed by culturing on PDA (26). Because culture morphology is an indicator of CHV-1 infection (8), *C. parasitica* isolates were determined to be virus-infected if they displayed the white culture morphology and to be virus-free if they displayed the orange morphology (22,28). Cankers from which only virus-free isolates were recovered were coded "O" (orange), cankers from which only virus-infected isolates were recovered were coded "W" (white), and cankers from which both virus-infected and virus-free isolates were recovered were coded "M" (mixed). These data were used to calculate the percentage of virus-free, virus-infected, and mixed cankers at each date investigated.

Based on the reisolations in September 2000, August 2004, and September 2008, we also determined whether the isolates recovered were of the same vc type as the original strains used for the sprout inoculations. Therefore, we paired the isolate and the original strain on the same PDA plate as described previously for vc type testing (5,11). If barrage formation was observed between the two colonies, the two isolates were regarded as vegetatively incompatible (i.e., of different vc type). If the two colonies merged without barrage formation, they were considered to be vegetatively compatible (i.e., sharing the same vc type). Cankers from which only vegetatively compatible isolates were recovered were coded "C" (compatible), cankers from which only vegetatively incompatible isolates were recovered were coded "I" (incompatible), and cankers from which both vegetatively compatible and incompatible isolates were recovered were coded "M" (mixed). The recovery of an isolate that was incompatible with the original strain indicated an immigration of a new fungal strain into the canker.

Data analysis. At each assessment date in both inoculation experiments, we calculated the mean canker area from the five replicates of each *C. parasitica* strain. The shape of the lesions

TABLE 1. *Cryphonectria parasitica* strains used for inoculation experiments

Name	Type ^t	CHV-1 ^u	Vc type ^v	Isolation ^w		Application ^x	
				Location	Year	Seedling experiment	Sprout experiment
51A	O-active	No	EU-2	Lumino	1994	Yes	Yes
76B	O-active	No	EU-5	Lumino	1994	Yes	Yes
303A	O-active	No	EU-6	Gnosca	1994	Yes	Yes
335B	O-active	No	EU-2	Gnosca	1994	Yes	No
357A	O-active	No	EU-5	Gnosca	1994	Yes	No
17A	O-inactive	No	EU-1	Lumino	1994	Yes	No
314A	O-inactive	No	EU-2	Gnosca	1994	Yes	Yes
323A	O-inactive	No	EU-1	Gnosca	1994	Yes	Yes
332A	O-inactive	No	EU-1	Gnosca	1994	Yes	No
356A	O-inactive	No	EU-5	Gnosca	1994	Yes	Yes
20A	W-active	Yes	EU-21	Lumino	1994	Yes	No
25A	W-active	Yes	EU-5	Lumino	1994	Yes	No
40A	W-active	Yes	EU-5	Lumino	1994	Yes	No
344A	W-active	Yes	EU-9	Gnosca	1994	Yes	No
338A	W-active	Yes	EU-15	Gnosca	1994	Yes	No
29A	W-inactive	Yes	EU-2	Lumino	1994	Yes	No
43A	W-inactive	Yes	EU-5	Lumino	1994	Yes	No
44A	W-inactive	Yes	EU-5	Lumino	1994	Yes	No
63A	W-inactive	Yes	EU-2	Lumino	1994	Yes	No
310A	W-inactive	Yes	EU-2	Gnosca	1994	Yes	No
M1115 ^y	O-control	No	EU-2	Gorduno	1976	Yes	Yes
M1095 ^z	W-control	Yes	EU-2	n.a.	n.a.	Yes	Yes

^t O-active = virus-free and isolated from active chestnut blight cankers; O-inactive = virus-free and isolated from inactive chestnut blight cankers; W-active = virus-infected and isolated from active chestnut blight cankers; W-inactive = virus-infected and isolated from inactive chestnut blight cankers. The letter O refers to the orange culture morphology of virus-free strains and the letter W refers to the white culture morphology of virus-infected strains.

^u *Cryphonectria hypovirus 1*.

^v Vegetative compatibility type (11).

^w All strains were obtained from southern Switzerland; n.a. = not applicable because strain was created in the laboratory.

^x Application in this study.

^y The virus-free strain M1115 was obtained from the fungal culture collection at WSL. It had been stored freeze-dried to avoid subculturing (25).

^z The virus-infected strain M1095 is isogenic with strain M1115 and was created in the laboratory by virus infection of strain M1115 (25).

resembled an ellipse; thus, we used the mathematical formula for an ellipse to calculate the size of the lesions. In the seedling experiment, we used the analysis of variance (ANOVA) in SPSS 19.0 (SPSS, Somers, NY) with strain type as the only factor to analyze the influence of the strain type (O-active, O-inactive, W-active, or W-inactive) on the canker area at a given time after inoculation. The Tukey's post-hoc test was implemented to detect significant differences ($\alpha < 0.05$) among strain types. Repeated-measures ANOVA was conducted with a reduced number of factors (i.e., orange versus white strains and strains from active versus inactive cankers). We also calculated the mean mortality caused by each *C. parasitica* strain and performed ANOVA and Tukey's test as described above. In the sprout inoculation experiment, we used ANOVA with strain type (O-active or O-inactive) as the only factor to detect significant differences ($P < 0.05$) between the strain types on the canker area at the given time after inoculation. We also performed a repeated-measures

ANOVA with the four time points to test for significant difference in canker growth between O-active and O-inactive strain types over time. The residuals of all ANOVAs in both experiments were normally distributed and displayed constant error variances.

RESULTS

Virulence of *C. parasitica* strains isolated from active and inactive chestnut blight cankers. In the chestnut seedling experiment, no significant differences were observed at all assessment dates between *C. parasitica* strains that had been recovered from active cankers and *C. parasitica* strains that had been recovered from inactive cankers, neither among virus-infected nor among virus-free strains (Table 2). This was true for both canker size and mortality of chestnut seedlings. Repeated-measures ANOVA also indicated that there was no significant difference in canker growth between strains recovered from active versus

Fig. 2. Callusing (left) and callused (right) chestnut blight cankers after artificial inoculation of *Cryphonectria parasitica* with a cork borer (see circular wound in the center of the callusing canker on the left). These pictures were taken in 2004, 9 years after inoculations, and the cankers were 16 (left) and 22 cm (right) long, respectively.

TABLE 2. Chestnut blight canker area on chestnut seedlings and chestnut seedling mortality induced by virus-free and virus-infected *Cryphonectria parasitica* strains isolated from active and inactive cankers

Type ^x	Canker area (cm ²) ^y			Mortality (%) ^z
	2 months	6 months	13 months	
O-active	35.38 ± 5.68 a	65.06 ± 17.95 a	146.37 ± 30.20 a	53 ± 18 a
O-inactive	31.42 ± 7.73 ab	75.42 ± 15.94 a	181.38 ± 34.98 a	52 ± 18 a
W-active	14.21 ± 8.87 b	20.93 ± 13.94 b	30.38 ± 23.37 b	5 ± 11 b
W-inactive	28.23 ± 14.53 ab	42.44 ± 23.79 ab	61.70 ± 33.99 b	8 ± 18 b

^x Four-year-old chestnut seedlings were inoculated on 26 June 1995 with virus-free (O) and virus-infected (W) *C. parasitica* strains isolated from active and inactive chestnut blight cankers.

^y Mean canker area ± standard deviation produced by each type at 2, 6, and 13 months after inoculation ($n = 5$). Values within the same column followed by the same letter are not significantly different according to Tukey's test at $\alpha < 0.05$.

^z Mean percentage of dead chestnut seedlings ± standard deviation. Values within the same column followed by the same letter are not significantly different according to Tukey's test at $\alpha < 0.05$.

inactive cankers over time (canker type by repeats: F ratio = 1.48; $P = 0.23$). However, significant differences in canker size and mortality were observed between virus-infected and virus-free strains (Table 2). The growth of cankers initiated by virus-

infected strains was significantly lower than the growth of cankers initiated by virus-free strains (strain-repeats: F ratio = 119.62, $P < 0.0001$). The differences in size between virus-free and virus-infected cankers increased over the course of time (Table 2). At

TABLE 3. Chestnut blight canker area on chestnut sprouts induced by virus-free and virus-infected *Cryphonectria parasitica* strains isolated from active and inactive cankers

Type ^x	Canker area (cm ²) ^y			
	6 months	15 months	30 months	39 months
O-active	83.79 ± 13.40 a	183.05 ± 78.03 a	208.59 ± 93.63 a	266.93 ± 140.87 a
O-inactive	67.35 ± 9.61 a	191.56 ± 93.37 a	342.62 ± 234.33 a	426.50 ± 296.26 a
O-control	79.92 n.a.	181.53 n.a.	202.78 n.a.	n.a. n.a. ^z
W-control	17.24 n.a.	22.66 n.a.	27.80 n.a.	27.33 n.a.

^x Eleven-year-old chestnut sprouts were inoculated on 26 June 1995 with virus-free *C. parasitica* strains isolated from active and inactive chestnut blight cankers. As controls, a virus-free (O) and a virus-infected (W) strain from the fungal culture collection at WSL were also used for infections.

^y The mean canker area ± standard deviation produced by virus-free (O)-active and O-inactive *C. parasitica* strains at 6, 15, 30, and 39 months after inoculation are presented ($n = 3$). For the controls, the canker area at each date is also presented. Values within the same column followed by the same letter are not significantly different according to the analysis of variance with $P < 0.05$; n.a. = not applicable.

^z By September 1998, two of the five sprouts infected with the O-control strain had died. Thus, the mean value for canker size in 1998 was not representative anymore and, therefore, is not presented.

TABLE 4. Virus infection of chestnut blight cankers over the course of 13 years

Canker number	Date ^z					
	1995 (inoculation)	March 1997	November 1997	2000	2004	2008
1	O	W	W	W	O	O
2	O	W	W	M	O	O
3	O	W	W	M	W	O
4	W	M	W	M	W	M
5	O	O	M	W	M	O
6	O	O	M	W	M	O
7	O	W	W	M	M	–
8	O	W	W	M	M	O
9	O	M	M	W	–	–
10	O	W	W	O	O	O
11	O	W	M	M	W	–
12	W	M	M	O	M	–
13	O	M	W	M	M	–
14	O	W	W	W	–	–
15	O	W	M	M	O	–
16	O	W	W	M	M	O
17	O	W	W	W	M	O
18	O	W	W	M	O	–
19	O	M	W	W	M	M
20	W	W	M	O	–	–
21	O	W	W	M	O	–
22	O	O	M	M	M	–
23	O	O	O	O	–	–
24	O	W	W	W	W	–
25	O	O	O	M	M	–
26	O	W	W	M	M	–
27	O	W	M	W	W	O
28	W	M	W	M	M	–
29	O	W	W	M	O	M
30	O	W	O	M	O	O
31	O	W	W	W	O	O
32	O	W	W	M	O	–
33	O	W	W	W	M	O
34	O	W	W	M	O	–
35	O	W	W	M	M	O
36	W	W	W	O	–	–
37	O	M	M	W	–	–
38	O	M	W	M	M	–
39	O	M	M	W	–	–
40	O	W	W	M	M	M
W (%)	12.5	65.0	65.0	32.5	14.3	0.0
M (%)	0.0	22.5	27.5	55.0	54.3	22.2
O (%)	87.5	12.5	7.5	12.5	31.4	77.8

^z Thirty-five 11-year-old *Castanea sativa* sprouts were inoculated with virus-free *Cryphonectria parasitica* strains and five sprouts were inoculated with virus-infected strains in June 1995. During the course of 13 years, virus infection of the formed chestnut blight cankers was determined at five points in time. At each time, three isolations (top, middle, and bottom of the canker) were made. If all isolates were virus free (i.e., orange), the canker was assigned the code “O”, if all isolates were virus-infected (i.e., white), the canker was assigned the code “W”, and if both virus-infected and virus-free isolates were obtained from the same canker, the canker was assigned the code “M” (i.e., mixed); – indicates dead sprouts or cankers from which isolations of *C. parasitica* were not successful. At the bottom of the table, the percent values of O, W, and M cankers at each point in time are presented.

the final assessment date, 13 months after inoculation, the mean canker area produced by the virus-infected strains was 2.3- to 6.0-fold smaller than the canker area produced by the virus-free strains. Similarly, the mortality of seedlings infected with virus-infected strains was <10%, whereas the mortality of seedlings infected with virus-free strains was >50% (Table 2). The two control strains behaved as expected. The O-control strain produced large cankers and high mortality and the W-control strain small cankers and no mortality (data not shown).

The same pattern was also observed in the chestnut sprout inoculation experiment. No significant differences in canker size (Table 3) and canker growth over time (strain–repeats: F ratio = 1.90, P = 0.14) were observed between *C. parasitica* strains that had been recovered from active versus inactive cankers at all dates investigated. As on the chestnut seedlings, the main differences in canker size were observed between the virus-free strains and the virus-infected control. These differences also increased over the course of time (Table 3).

Dynamics of virus infection and invasion of foreign vegetative compatibility types. Thirty-five cankers had been initiated by virus-free *C. parasitica* strains on chestnut sprouts. From all these cankers, virus-infected strains were isolated at least once between inoculation in 1995 and the end of the observation period in 2008 (Table 4). From the five initially virus-infected cankers, virus-free strains were also isolated at later time points. Virus presence in cankers was very dynamic and the proportions of virus-infected, virus-free, and mixed cankers differed over the course of 13 years. At the beginning, most cankers (87.5%) were initiated by virus-free strains. Two years after inoculation all strains recovered from the majority of cankers (65.0%) were virus infected. At 5 to 9 years after inoculation the majority of cankers (51.5 to 55.0%) were mixed (i.e., both virus-infected and virus-free strains were recovered). Finally, 13 years after infection, all cankers on surviving sprouts were inactive cankers (as described below) but, from most cankers (77.8%), only virus-free strains were recovered (Table 4). Of the 40 sprouts observed in this study, 12 died over time: six due to *C. parasitica* infection and nine due to other reasons (mostly competition among sprouts within the same cluster).

The number of cankers from which only the original vc type was isolated decreased over time (Table 5). Thirteen years after infection, less than half of all cankers (33.3%) only harbored the original vc type. Accordingly, the proportion of cankers harboring foreign vc types increased and reached 66.7% after 13 years.

Morphological development of chestnut blight cankers. Two years after inoculation, only 26% of the cankers on living sprouts were active, 61% were callusing, and 13% were callused (Fig. 3). Over time, the number of active cankers decreased gradually. In 2004 and 2008 (i.e., 9 and 13 years after inoculation), no active cankers were observed anymore. In 1999, the number of callusing cankers also started to decrease. This was associated with an increase in the number of callused cankers (Fig. 3). At the end of the assessment period in 2008, all cankers on surviving sprouts were fully callused, except for two cankers that were still callusing.

DISCUSSION

In this study, we tested the hypothesis that virus-free *C. parasitica* strains isolated from inactive chestnut blight cankers are less virulent than virus-free *C. parasitica* strains isolated from active cankers. However, we found that these strains did not significantly differ in virulence as assessed by canker growth and host mortality in inoculation experiments. The only difference in virulence was observed between virus-infected and virus-free strains, regardless of whether they were obtained from active or inactive cankers.

C. parasitica strains isolated from active cankers did not produce larger lesions than strains isolated from inactive cankers.

Likewise, these strains did not lead to a higher mortality rate of chestnut seedlings. Significant differences in virulence were observed between virus-infected and virus-free strains, as expected. Virus-infected strains produced significantly smaller lesions than virus-free strains on chestnut seedlings and sprouts. These differences in lesion size became more accentuated over time. In addition, virus-infected strains caused significantly lower, nearly marginal mortality rates in chestnut seedlings. It is unlikely that differences in susceptibility or resistance among chestnut trees

TABLE 5. Presence of different vegetative compatibility types of *Cryphonectria parasitica* in chestnut blight cankers over the course of 13 years

Canker number	Date ^y			
	1995 ^z	2000	2004	2008
1	EU-2	C	M	I
2	EU-5	M	I	I
3	EU-6	M	C	C
4	EU-2	C	C	M
5	EU-2	C	M	I
6	EU-1	I	I	M
7	EU-2	I	I	–
8	EU-5	M	M	M
9	EU-2	C	–	–
10	EU-5	C	I	M
11	EU-6	M	C	–
12	EU-2	C	M	–
13	EU-2	M	M	–
14	EU-1	C	–	–
15	EU-2	M	M	–
16	EU-5	M	C	M
17	EU-2	C	C	C
18	EU-5	C	I	–
19	EU-6	M	C	M
20	EU-2	C	–	–
21	EU-2	C	C	–
22	EU-1	C	C	–
23	EU-2	C	–	–
24	EU-5	C	C	–
25	EU-2	C	C	–
26	EU-5	M	I	–
27	EU-6	C	C	I
28	EU-2	C	M	–
29	EU-2	M	M	C
30	EU-1	C	M	I
31	EU-2	C	C	C
32	EU-5	C	C	–
33	EU-2	C	nd	C
34	EU-5	C	C	–
35	EU-6	C	C	M
36	EU-2	C	–	–
37	EU-2	C	–	–
38	EU-1	C	C	–
39	EU-2	C	–	–
40	EU-5	C	C	C
I (%)	n.a.	5.0	18.8	27.8
M (%)	n.a.	25.0	28.1	38.9
C (%)	n.a.	70.0	53.1	33.3

^y Forty 11-year-old *Castanea sativa* sprouts were infected with *Cryphonectria parasitica* strains of different vegetative compatibility (vc) type (EU-1, EU-2, EU-5, or EU-6) in June 1995. During the course of 13 years, the presence of different vc types in formed chestnut blight cankers was determined at three points in time. At each time, three isolations (top, middle, and bottom of the canker) were made. If all isolates were of the same vc type as the original isolate, the canker was assigned the code “C” (i.e., compatible); if all isolates were of a different vc type than the original isolate, the canker was assigned the code “I” (i.e., incompatible); and if isolates of both the same and of different vc types were obtained from the same canker, the canker was assigned the code “M” (i.e., mixed); – indicates dead sprouts or cankers from which isolations of *C. parasitica* were not successful; nd = not determined due to contamination of the isolated fungal culture the vc type. At the bottom of the table, the percent values of C, I, and M cankers at each point in time are displayed; n.a. = not available.

^z Year of inoculation, vc type.

were responsible for the observed morphology and mortality. In the seedling inoculation experiment, all seedlings were offspring of the same mother tree and, thus, large differences in susceptibility or resistance were not expected. In the sprout inoculation experiment, all sprout clusters were situated in the same forest stand. It had been shown previously that little variation in susceptibility or resistance was present within the chestnut populations in the area where the experiment was conducted (4). Therefore, differences in morphology and mortality can mainly be attributed to hypovirulence induced by virus infection.

In this and in previous studies (5,8,23), we isolated virus-free strains from inactive cankers. Here, we investigated the dynamics of virus infection in chestnut blight cankers. Therefore, we artificially initiated 35 virus-free and 5 virus-infected cankers in a chestnut stand with naturally occurring hypovirulence and then periodically assessed the presence of CHV-1 in these cankers over the course of 13 years. During the examination period, each of the cankers was virus infected on at least one assessment date. All cankers changed between virus-free and virus-infected states. Similar observations had been made previously for naturally occurring chestnut blight cankers in the same region (5). Within the first 2 years after infection, virus infection increased rapidly and CHV-1 was soon found in the vast majority of cankers. In the following 11 years, however, virus infection decreased gradually to very low numbers. Nevertheless, all cankers on surviving sprouts were inactive (callused or callusing) at the end of the examination period. No active cankers were observed 9 years after inoculation and onwards.

The *Cryphonectria*–hypovirus pathosystem is a textbook example for a natural biological control system that has been very successful (1,14). In extensively managed ecosystems, such as forests, biocontrol agents should establish in the long term, exerting sufficient control without the need for recurrent human interventions (17,21). Therefore, suitable biocontrol agents are expected to spread independently after initial treatments. The results of this study demonstrate the effectiveness of CHV-1 dissemination within a forest with naturally established hypo-

virulence without human help. In our study, all initially virus-free cankers became naturally virus infected within a short time period. Therefore, virus infections of cankers initiated by virus-free *C. parasitica* strains seem to occur rapidly if virus-infected inoculum is present in the population.

Our results suggest that callusing of cankers and cessation of canker expansion is directly attributed to virus infection. However, CHV-1 seems to not persist in these inactive cankers and, thus, to disappear over time. A potential explanation for this lack of persistence of CHV-1 in inactive cankers may be found in the histopathology of virus-infected cankers. Callusing of virus-infected cankers is associated with cessation of canker expansion (13) and, finally, inhibits the growth of *C. parasitica* completely. The containment and inactivation of *C. parasitica* in these cankers could lead to the disappearance of CHV-1 from the fungal mycelium. CHV-1 is an obligate parasite and completely dependent on the metabolism of its fungal host (20). Therefore, it requires an active fungal mycelium to maintain a significant virus concentration. This could explain why CHV-1 cannot always be isolated from inactive, previously virus-infected cankers. Such difficulties in isolating CHV-1 from inactive cankers have been experienced before (8,18,23). However, it is important to note that the *C. parasitica* strains contained in inactive cankers do not differ in virulence from *C. parasitica* strains present in active cankers. When isolated and inoculated onto fresh seedlings or sprouts, these virus-free strains are all highly virulent.

This finding may have implications for forest management. It has been reported that *C. parasitica* strains contained in inactive cankers may resume their activity after the tree or sprout had been cut or weakened after fire, drought, or death due to competition (23). *C. parasitica* from inactive cankers started to grow and sporulate abundantly as a saprophyte on the dying host tissue for at least 1 to 2 years. Foresters and chestnut orchard managers should take this possibility into account when cutting or pruning chestnut sprouts or trees. On the one hand, inactive cankers on recently cut sprouts or stems may serve as a source of hypovirulent, virus-infected inoculum if CHV-1 is still present in the

Fig. 3. Morphology of chestnut blight cankers (active, callusing, or callused) and dead chestnut sprouts (dead due to *Cryphonectria parasitica* infection = dead_CP or dead due to other reasons = dead_NOTCp) over the course of ≈11 years. The total number of chestnut sprouts was 40.

fungal mycelium (23). This would be favorable for the biological control of chestnut blight because it would promote the natural dissemination of CHV-1. On the other hand, in cases where CHV-1 had disappeared from the fungal mycelium over time, the opposite effect would be observed. Inactive cankers on recently cut chestnut wood could become a source of virulent, virus-free chestnut blight inoculum. This could eventually lead to an increase in both disease incidence and severity. The importance of such virulent sporulation on cut or weakened sprouts or trees for the sustainability of the biocontrol system should be further investigated. Our results indicate that the chances of disappearance of CHV-1 from inactive cankers increased with increasing age of the canker. As a forest health management practice, therefore, the removal of the cut sprouts or trees from the site should be well considered if they are associated with inactive cankers.

The dynamic nature of the *Cryphonectria*–hypovirus pathosystem is illustrated not only by the variability in virus infection described above but also by the immigration of foreign vc types into chestnut blight cankers. In our study, the percentage of cankers from which vc types incompatible with the original vc type were recovered increased over time and affected the majority of cankers 13 years after inoculation. This shows that not only the virus presence but also the fungal strain composition changes over time in a canker. Chestnut blight cankers seem to be an open system, serving as both a source and sink for fungal and viral inoculum. Invasion of existing cankers by either virus-free or virus-infected fungal individuals and transmission of CHV-1 among these individuals results in a dynamic fungus–virus system. Therefore, we suggest that studies not focus on virus infection and *C. parasitica* strain composition within individual cankers. Instead, incidence of virus infection and fungal strain composition within a population or forest should be used to assess the overall disease severity. Under the presence of a sufficient virus incidence within the population, a low disease severity will most likely be maintained over time.

In conclusion, our study showed that the *Cryphonectria*–hypovirus pathosystem is a very dynamic but effective biocontrol system. Virus infection and fungal strain composition of chestnut blight cankers change over time. Under the presence of natural hypovirulence in the area, virus-free cankers become virus-infected within a few years, leading to inactive cankers. In these cankers, the virus might not persist over time. Therefore, inactive cankers can also harbor virus-free strains of *C. parasitica*. The virulence of these virus-free strains is not naturally low when inoculated onto living seedlings or sprouts. They are as virulent as virus-free strains isolated from active cankers. These findings are important for forest health management. On the one hand, CHV-1 seems to disseminate efficiently within a population, leading to hypovirulence and healing of cankers. On the other hand, old, inactive cankers may serve as a source of virulent, virus-free chestnut blight inoculum due to the saprophytic activity of *C. parasitica* in freshly cut trees. These factors should be considered in forest management practices as well as in biological control programs.

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