Relationship Between Biological Control, Incidence of Hypovirulence, and Diversity of Vegetative Compatibility Types of *Cryphonectria parasitica* in France

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ABSTRACT

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In France, chestnut blight, caused by *Cryphonectria parasitica*, has been controlled since 1974 in orchards, but never in coppice forests, by releasing hypovirulent strains infected with CHV1 hypovirus. We tested the hypothesis that this biological control (BC) has led to a decrease in blight severity, spread of hypovirulence, and change in *C. parasitica* populations. The low severity of chestnut blight was confirmed in the six regions studied (subdivided into zones). The remission of cankers was associated with the presence of white isolates presumed to be hypovirulent. These two parameters were also correlated, at the zonal level, to the frequency of sites where BC was used. However, the

estimates of the natural background level of hypovirulence, independent of BC, ranged from 4% in forests in Dordogne to 60% in orchards in Lozère. Differences in the rate of hypovirulent isolates among regions were consistent with the diversity of vegetative compatibility (VC) types in populations of *C. parasitica*. The highest VC-type diversity and mean allelic diversity for known vegetative incompatibility (*vic*) genes were observed in Dordogne. We showed that the current diversity of VC types in populations of *C. parasitica* was lower than in 1981. We found 30 VC types among 1,113 isolates of *C. parasitica*. Ten VC types were incompatible with known EU testers, suggesting that one additional *vic* gene or allele at one of the six *vic* loci known should be present in Europe.

Additional keywords: *Castanea sativa*, *Endothia parasitica*, epidemiology, population differentiation.

The hypovirulence caused by *Cryphonectria hypoviruses* (CHVs) on *Cryphonectria parasitica* (Murr.) Barr, the causal agent of chestnut blight, is an example of virus-mediated attenuation of pathogen virulence (11,16,24,41). Infections by hypovirulent isolates result in superficial cankers on both European (*Castanea sativa* Mill.) and American (*C. dentata* [Marsh] Borkh) chestnut trees, whereas virus-free isolates cause deep, lethal cankers. CHVs are cytoplasmic double-stranded RNA viruses that move into conidia, but not into ascospores, and can be transmitted from an infected isolate to a virus-free isolate through hyphal anastomosis. Hypovirulence offers great potential for biological control (BC) of chestnut blight as stated in 1965 by Grente (18) and reviewed later by several authors (7,23,32,44,45). In France, the Ministry of Agriculture has promoted an intensive release of hypovirulent isolates for BC of chestnut blight on 180,000 ha since 1974. Today BC is still applied in orchards, especially in southeastern France and to a lesser extent in southwestern France (Dordogne and Corrèze), but never in coppice forests. BC was applied following Grente's protocol (20); in each district, up to eight hypovirulent isolates, selected according to the vegetative compatibility (VC) types present in the populations of *C. parasitica* (19,20), were released. The selected mixtures of hypovirulent isolates were prepared by a private company and first applied by specialized technicians and later by growers. Every year in these orchards, all newly formed cankers are treated with hypovirulent isolates by introducing the mycelia

into holes in the margin of the canker. This treatment has a curative effect and favors the healing of the canker when the virus is successfully transmitted. BC may have a preventative effect depending on the spread of hypovirulent strains within the area where they were released. According to Grente, the percentage of healing cankers increased in orchards treated with BC (19) and a similar result was obtained in Italy (8). A better knowledge of the spread of hypovirulence is crucial to predict the efficacy of BC and the dynamics of blight incidence in chestnut ecosystems.

Although hypovirulent isolates with broad conversion capacities were found (28,34), vegetative incompatibility restricts hypovirus transmission. The rate of transmission is negatively correlated with the number of vegetative incompatibility (*vic*) genes that differ between mycelia that anastomose (2,25,31). The spread of CHVs should be directly correlated to the quantity and effectiveness of inoculum produced by hypovirulent isolates and to the population genetic structure for VC types. A high percentage of virus-infected isolates should be found in populations with low diversity of VC types, limiting chestnut blight severity. At the continental level, this scenario fits the lack of spread of hypoviruses in populations of *C. parasitica* in North America, characterized by high VC diversity (4,5,32,41). By contrast, in Europe the low VC-type diversity favors the spread of hypovirulent isolates of *C. parasitica* and the recovery of European chestnut in many areas (22). In Switzerland, the rate of hypovirulent isolates of two fungal populations was associated with low VC-type diversity (10). However, few studies have associated diversity of VC types and spread of hypovirulence on a smaller scale, i.e., in different populations of *C. parasitica*. In France, spontaneous healing of cankers was reported recently by chestnut growers, suggesting possible local spread of hypovirulence.

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Hypovirus CHV1 is responsible for debilitation, reduced sporulation, and female sterility of infected strains (3,11,18,44,47). Consequently, the spread of CHV1 in a fungal population may have a significant effect on the reproductive behavior of *C. parasitica* (35). Thus, the frequent release of hypovirulent strains for BC may cause a significant change in population structure for VC-type diversity, canker morphology, and proportion of white isolates. We tested the hypothesis that the regression of chestnut blight incidence due to BC has lowered the VC-type diversity found in France in 1981 as reported by Greute (19), the only study available. We also studied the distribution and diversity of VC types in populations of *C. parasitica* from the main chestnut growing areas of France.

MATERIALS AND METHODS

Study sites, sampling strategy, and *C. parasitica* isolation.

C. parasitica was sampled from 1995 to 1997 in the most economically important chestnut-growing regions in France: in orchards in Ardèche, South Corsica, Gard, and Lozère, in orchards and coppice forests in Languedoc-Roussillon, and in coppice forests in Dordogne. In Ardèche the chestnut cover was mostly continuous, whereas in the other regions all zones sampled were separated either by relief or other land-use patterns (Fig. 1). Zones LR-1, LR-2 and LR-3 were located in three districts, i.e., Aude,

Pyrénées Orientales, and Hérault, respectively. In each orchard, the history of BC was investigated by interviewing the grower to determine whether and when hypovirulent isolates were released for BC. The intensity of BC in zones and regions was assessed based on the percentage of sites where BC was applied at least once (BCS). The *C. parasitica* isolates obtained in each zone were pooled to constitute the subpopulations, and the subpopulations of each region were pooled into one population per region. In each zone, *C. parasitica* was sampled from several chestnut sites, with an average distance of 5 km between sites. In each site, at least 10 evenly distributed blighted trees (neighbor trees were avoided as far as possible) were chosen, except in Dordogne where only 5 trees were chosen, and one canker per tree was sampled to avoid clones (38). Cankers that were treated with hypovirulent isolates were not sampled. Each canker sampled, except in Dordogne in 1996, was scored for morphology and was classified as active, healing, or healed. From each canker five bark samples, four from the edges and one from the middle, were removed with a cork borer (8 mm in diameter) and stored in a plastic tube until isolation. Bark samples were surface-disinfected for 15 s in 70% ethanol, rinsed in sterile water, blotted on filter paper, and placed on potato dextrose agar (PDA at 39g/liter, Difco Laboratories, Detroit). One isolate per canker was randomly chosen and transferred to PDA. To avoid bias by selecting isolates based on mycelium color, this was done before pigmentation occurred, i.e.,

Fig 1. Regions and zones of France where *Cryphonectria parasitica* was sampled.

after 3 days of incubation at 25°C in the dark. Mycelium color of each isolate was scored after 8 days of incubation at 25°C and a 16-h photoperiod. Isolates with a white phenotype were termed white, and were presumed to be infected with CHV1 (11,16), whereas isolates with an orange phenotype were presumed to be virus-free. All isolates were stored in tubes on PDA slants at 6°C.

VC and conversion tests. VC was evaluated on PDA supplemented with colorant, either amaranth (Sigma Chemical Company, St. Louis) or bromocresol green, sodium salt (Research Organics Inc., Cleveland, OH) at a final concentration of 120 and 50 mg/liter, respectively, to enhance the barrage reaction between incompatible isolates (25,42). Small cubes of 7-day-old agar-mycelium were paired $\approx$ 1 mm apart, 1 cm from the edge of a 9-cm petri dish. Six pairings were made per petri dish. Dishes were incubated for 7 days in the dark at 25°C, followed by 7 days incubation in daylight. Vegetative incompatibility was scored after 7 and 14 days of incubation. Isolates were considered incompatible when a barrage was observed between the two mycelia; they were considered compatible when the two mycelia merged completely (4). All isolates from each subpopulation were first paired in all combinations, and one isolate of each VC type was kept as a tester. The testers of all subpopulations were successively paired in all combinations to find the compatible VC types, and finally, one tester isolate for each VC type was paired, at least twice, against the set of 64 European testers (EU) (13,15) and against the Swiss isolate CH-27, named EU-65 by D. Rigling (Swiss Federal Institute for Forest, Snow, and Landscape Research, Birmensdorf, *personal communication*; Table 1). The pairings with testers EU-1 to EU-31 and EU-65 were performed in France, while in Italy the isolates found incompatible were paired on PDA (14) with all 65 EU testers. Conversion tests were conducted as previously described (19) by pairing each of the six hypovirulent CHV1-infected isolates used in France for BC (Table 1) with all EU testers for VC types found in the French population of *C. parasitica*. Change in the mycelium color of the EU testers, from orange to white, was taken as evidence of hypovirus transmission and conversion to hypovirulence.

Assessment and comparison of VC-type diversity. The Shannon index ($H_s = -\sum p_i (\ln p_i)$, $i = 1, \dots, S$, where p_i is the frequency of the i th VC type and S is the number of VC types) was calculated for each population and subpopulation. This diversity index followed a normal distribution (33). A t -test described by Hutcheson (26) allows the comparison of Shannon

indices between two populations. The value of t was calculated according to the formula

$$t = (H_{s1} - H_{s2}) / (\text{Var}H_{s1} + \text{Var}H_{s2})^{1/2} \text{ with } \text{Var}H_s = [\sum p_i (\ln p_i)^2 - (\sum p_i \ln p_i)^2 / N - (S - 1) / (2N^2)]. \text{ The number of degrees of freedom (df) was calculated as}$$

$$\text{df} = [(\text{Var}H_{s1} + \text{Var}H_{s2})^2 / ((\text{Var}H_{s1})^2 / N_1 + (\text{Var}H_{s2})^2 / N_2)].$$

The method developed by Lewontin (29) to partition diversity into hierarchical and geographical components using the Shannon index was applied to our sample. The two hierarchical levels studied were zones and regions. Shannon diversity indices calculated for each zone, each region, and France, using the VC-type frequencies calculated for each subpopulation, each population, and for total sample, respectively. The total diversity was allocated to the between-region, between-zone, and within-zone components.

Using the *vic* genotypes (13) of the EU types found in France, allelic diversity for each *vic* locus and mean allelic diversities for each population were estimated as described by Milgroom and Cortesi (36).

RESULTS

Frequency of chestnut sites treated with BC and canker morphology. The frequency of BC was estimated in each region and zone (Table 2). In Gard, Lozère, and Ardèche, BC was applied regularly in a high percentage of orchards, i.e., more than 50%. The first release of hypovirulent isolates dated back to 1978 in these regions. BC was applied at different frequencies within regions (e.g., in Ardèche). In Corsica and Languedoc-Roussillon, the percentage of BCS was lower, and BC was applied more sporadically than in the latter regions. The neglect of chestnut groves resulted in a low percentage of BCS in Cor-4. In Dordogne, the percentage of BCS was nil because BC is not applied in coppice forests.

In each zone, *C. parasitica* was isolated from 77 to 100% of cankers sampled. Isolation failure was higher for healed cankers than for active or healing cankers (results not shown). Only cankers yielding *C. parasitica* were retained for further analyses. In all regions, the percentage of cankers in remission (healing or healed) was higher than 30% (Table 2). However, there were significant differences in the frequency of the three types of cankers among regions ($\chi^2 = 307.7$, $P < 1\%$). The percentage of healed cankers was nil in Dordogne, 22% in Lozère, and less than 10% in the other regions. In Ardèche and Lozère, less than 30% of cankers were active whereas in Corsica, Languedoc-Roussillon, and Dordogne more than 60% of the cankers were active. The percentage of BCS in each zone was significantly correlated with the percentage of cankers in remission (Pearson coefficient: $R = 0.522$, $P < 1\%$).

Recovery of white isolates. Altogether, 25% of the 1,113 isolates had a white phenotype. They were obtained from healed, healing, and active cankers (Table 3). In Ardèche and South Corsica a similar percentage of cankers yielded white isolates regardless of canker morphology. In Gard, however, a higher percentage of white isolates was obtained from healing or healed cankers. At the regional level, the percentage of white isolates varied significantly, from 4% in Dordogne to 11% in South Corsica, and 59% in Lozère (Table 2). At the zonal level, the percentage of white isolates was correlated with the percentage of BCS and the percentage of cankers in remission (Pearson coefficients; $R = 0.584$ and 0.580 , respectively; $P < 1\%$). However, white isolates were found in two zones, Cor-4 and Dor-1, where hypovirulent isolates were not released (Table 2). Conversely, in Cor-3, where hypovirulent isolates had been released in three of

TABLE 1. Origin of isolates of *Cryphonectria parasitica* used as vegetative compatibility testers and for biological control of chestnut blight

Isolates	Origin
EU-1 to -31	Cortesi et al. 1998 (15)
EU-32 to -64	Cortesi and Milgroom 1998 (13)
EU-65	D. Rigling: CH-27 Ollon, Vallais, Switzerland, 1997
EU-66	This study: MSD3, Fossemagne, Dordogne, France, 1995
EU-67	This study: MSA10, Pont de Beaume, Ardèche, France, 1995
EU-68	This study: MSA3, St. Clerge la Serre, Ardèche, France, 1995
EU-69	This study: MSA9, Chassiers, Ardèche, France, 1995
EU-70	This study: MSD14, St. Caprais, Dordogne, France, 1995
EU-71	This study: D15, Bourdeix, Dordogne, France, 1995
EU-72	This study: MSD5, Issac, Dordogne, France, 1995
EU-73	This study: MSD12, St. Laurent La vallée, Dordogne, France, 1995
EU-74	This study: D2, Firbeix, Dordogne, France, 1995
2022	Grente, Gonfaron, Var, France, hypovirulent, CHV1 infected, 1970
2079	Grente, Gonfaron, Var, France, hypovirulent, CHV1 infected, 1970
2100	Grente, Sablières, Corrèze, France, hypovirulent, CHV1 infected, 1975
2103	Grente, Castillon, Hautes Pyrénées, hypovirulent, France, CHV1 infected, 1975
2113	Grente, Ste. Fortunée, Corrèze, France, hypovirulent, CHV1 infected, 1975

five sites, only 7% of white isolates were recovered. On a finer scale, the percentages of white isolates in sites with BC and sites without BC were not significantly different (Fig. 2), except in Gard where a significantly higher percentage of white isolates was recovered in sites where hypovirulent isolates were released ($\chi^2 = 4.406$, $P = 3.6\%$). Most of the white isolates belonged to VC types EU-2, EU-1, and EU-5. In our tests, the BC agents did not have a broad conversion capacity, each converting only two or three VC types.

Diversity of VC types in populations of *C. parasitica*. The Shannon diversity index ranged from 2.05 for Dordogne to 1.11 for Corsica (Table 2). Among the 132 isolates from Dordogne, a total of 14 VC types was detected, with the 4 most frequent types, EU-33, EU-2, EU-72, and EU-66, accounting for 31, 17, 12, and 10% of isolates, respectively (Fig. 3). In contrast, in Corsica, Gard, Lozère, and Languedoc-Roussillon EU-2 was gathered from 50 to 70% of the isolates, and only three VC types (EU-2, EU-1, and EU-5) were gathered from more than 81% of the isolates. In Ardèche, VC-type EU-2 represented 67% of the isolates, and VC types EU-65, EU-1, and EU-68 represented between 6 and 10% of the isolates. According to the *t*-test performed on *Hs*, the

Dordogne population was significantly different from all other populations ($t > 6$, $P < 1\%$), and the Corsican population was significantly different from the populations of Lozère ($t = 2.50$, $P < 1\%$) and Gard ($t = 1.96$, $P = 5\%$). The partition of the Shannon diversity index into hierarchical components confirmed the differentiation at the regional level, as well as at the zonal level. The between-region, between-zone, and within-zone components accounted for 23.4, 13, and 66.3%, respectively, of the total

TABLE 3. Percentage of cankers yielding white isolates of *Cryphonectria parasitica* in southeastern regions of France^a

Region	Canker type		
	Active	Healing	Healed
Ardèche	23	21	13
Corsica	11	12	15
Gard	29	53	80
Languedoc- Roussillon	16	23	50
Lozère	50	62	60

^a In Dordogne, morphology of cankers was assessed only in 1995, and white isolates were not obtained.

TABLE 2. Characteristics of chestnut zones and regions studied in France and diversity of vegetative compatibility (VC) types of resident subpopulations and populations of *Cryphonectria parasitica*

Zone	No. of sites	% of BCS ^a	% of HC ^b	No. of isolates	No. of VC types	<i>P</i> ^c	<i>Hs</i> ^d	% white ^e
Ard-1	6	75	77	56	4	0.77	0.78	39
Ard-2	5	80	69	49	5	0.74	0.85	20
Ard-3	3	33	76	29	5	0.59	1.03	3
Ard-4	4	100	83	37	4	0.78	1.00	27
Ard-5	5	40	76	44	4	0.39	1.26	2
Ard-6	4	50	95	39	4	0.72	0.88	21
Ard-7	5	20	86	49	5	0.65	1.03	16
Ard-8	6	33	69	57	6	0.81	0.78	26
Cor-1	5	20	30	46	4	0.70	0.89	9
Cor-2	5	40	44	49	3	0.49	0.77	16
Cor-3	5	80	40	41	5	0.66	0.98	7
Cor-4	5	0	41	45	5	0.56	1.07	11
Dor-1	12	0	42	51	9	0.35	1.20	10
Dor-2	9	0	27	42	10	0.36	1.93	0
Dor-3	8	0	30	39	9	0.39	2.41	0
Gard-1	7	86	56	59	6	0.67	1.03	40
Gard-2	6	83	53	41	6	0.44	1.70	34
Gard-3	6	75	49	52	8	0.31	1.50	25
LR-1	2	50	50	19	3	0.53	0.94	60
LR-2	4	50	50	32	4	0.38	1.32	6
LR-3	7	29	49	64	5	0.83	0.66	25
Loz-1	4	75	94	35	6	0.43	1.20	63
Loz-2	6	100	88	46	7	0.37	1.60	41
Loz-3	5	60	98	40	5	0.48	1.19	65
Loz-4	6	83	98	52	8	0.67	1.13	67
Region								
Ardèche	38	53	55	360	10	0.68	1.18	21
Corsica	20	35	39	181	6	0.61	1.11	11
Dordogne	29	0	31	132	14	0.30	2.05	4
Gard	19	84	55	152	12	0.55	1.49	33
Lozère	21	81	40	173	11	0.50	1.46	59
LR	13	38	49	115	7	0.65	1.12	25
Total	140	46	49	1,113	30	0.63	1.75	25

^a Number of sites where biological control of chestnut blight was applied (BCS).

^b Percentage of healing or healed cankers (HC).

^c Frequency of the most common VC type.

^d Shannon index for VC type diversity.

^e Percentage of white isolates of *C. parasitica*.

diversity. When the population of Dordogne was omitted, the estimates of the three components were 6, 10.6, and 85.3%, respectively. On average, about six VC types were detected per subpopulation (Table 2). In 15 subpopulations of 25, the dominant VC type included more than 50% of the isolates. Because of the differentiation of *C. parasitica* between regions, the relationship between the spread of white isolates and the diversity of *C. parasitica* subpopulations, as measured by the Shannon index,

was not analyzed across the 25 zones but was examined per region. Within each region, except Languedoc-Roussillon, the percentage of white isolates decreased as diversity increased. In Ardèche, the Pearson correlation coefficient between *Hs* and percentage of white isolates was significantly negative ($R = -0.81$, $P < 1\%$). In the other regions, the number of zones was too low to calculate the correlation coefficient.

In 1981, 20 VC types had been reported among the 138 isolates collected in 18 French districts (5,19). Among these VC types, VC types U.S. VC-12, VC-40 and VC-56, which are compatible with EU-2, EU-5, and EU-12, respectively (13,15), represented 3, 14, and 1% of the isolates, respectively (S. L. Anagnostakis, Connecticut Agricultural Experiment Station, New Haven, *personal communication*). Of 138 isolates, 7 were from Dordogne, 10 from Ardèche, 6 from Corsica, 10 from Gard, 7 from Lozère, and 9 from Hérault. When these 49 isolates were pooled, and 12 VC types were identified, VC types EU-2 and EU-5 represented 4 and 22% of the isolates, respectively, and *Hs* was 2.24. To compare previous results to ours, 10 subsets of 49 isolates were randomly drawn from our total sample, with the same number of isolates pooled per district studied as in Grente's study (19). In the 10 subpopulations sampled, the number of VC types varied from 8 to 12, and the mean frequency of VC types EU-2, EU-1, and EU-5 was 58.4, 14, and 6.1%, respectively. The Shannon diversity index varied from 1.31 to 1.63, with a mean value of 1.46, lower

Fig. 2. Distribution and frequency of vegetative incompatibility types of *Cryphonectria parasitica* in six regions of France.

Fig. 3. Percentages of white isolates of *Cryphonectria parasitica* obtained from sites where biological control of chestnut blight was or was not applied.

than the 2.24 diversity index calculated for the population sampled in 1981.

When all 1,113 isolates were pooled, 30 VC types were detected in *C. parasitica* populations in France (Table 4). Eighteen were assigned to VC types already described in Europe, and two, EU-33 and EU-38, were found for the first time in the field. Nine new VC types, i.e., VC types different from all 65 EU VC types, were named with numbers higher than 65 (Table 1) and, so far, have been found only in France. Among them, five VC types, EU-66, EU-70, EU-71, EU-73, and EU-74, were detected only in Dordogne, and three, EU-67, EU-68, and EU-69, only in Ardèche, whereas EU-72 was present in Dordogne and Languedoc-Roussillon. Two new VC types, EU-69 and EU-73, were represented by one isolate (Table 4), whereas the others consisted of several isolates from different sites within the region. The VC types EU-67 and EU-68 were represented by orange and white isolates and EU-69 only by a white isolate. In the whole sample, the most common VC type was EU-2, accounting for 56% of the isolates sampled. EU-1 and EU-5 were the second and third most common types, accounting for 13 and 7% of the isolates,

respectively. The remaining 27 VC types did not account for more than 5% of the isolates, and 6 were represented by a single isolate. White isolates of *C. parasitica* were distributed in 13 of the 30 VC types found (EU-1, EU-2, EU-5, EU-11, EU-12, EU-14, EU-25, EU-30, EU-33, EU-38, EU-67, EU-68, and EU-69). Each of the hypovirulent strains used for BC in France converted two or three VC types (Table 1). All together, 85% of the isolates could be converted by at least one hypovirulent strain (Table 4).

Allelic diversities were calculated for the six populations using only the VC types with a known *vic* genotype (Table 5). Allele 2 at *vic3* is rare since it is present only in three VC types, EU-10, EU-33, and EU-38, resulting in the lowest mean gene diversity. The VC types EU-10 and EU-38 were both found in Ardèche but were represented by only 4 isolates, whereas EU-33 was represented by 2, 5, and 40 isolates in Gard, Lozère and Dordogne, respectively. Mean allelic diversities ranged from 0.384 in Dordogne to 0.081 in Ardèche. The Dordogne population had six polymorphic loci whereas in southeastern populations the number of polymorphic *vic* loci varied from two to five. In these populations, a high percentage of isolates belonged to VC-type EU-2, which has only one *vic* gene that is different from VC types EU-1, EU-4, and EU-14 and two *vic* genes different from VC-type EU-5. Recombination between these types would not give an important increase in allelic diversity for *vic* loci. In contrast, in Dordogne the two most common VC types, EU-33 and EU-2, differed by four *vic* genes, therefore sexual reproduction can result in 14 additional VC types.

DISCUSSION

Our study provides the first assessment of chestnut blight severity in France. We assessed morphology and spread of hypovirulence as measured by the percentage of white isolates of *C. parasitica*, which are highly, but not always, associated with hypovirulence (23). As expected, healed cankers yielded white isolates, with a frequency ranging from 13 to 80%. However, white isolates were also recovered from 11 to 50% of active cankers in all regions, except Dordogne. These results may be due to our sampling method, which retained only one isolate from each canker, or to the coexistence of multiple isolates in cankers (10). The percentage of cankers in remission and the percentage of white isolates were correlated to each other, suggesting that the decrease in chestnut blight severity was associated with the spread of hypovirulence. Moreover, both percentages were correlated to the frequency of sites where BC was applied, suggesting that the spread of hypovirulence is associated with the use of BC. However, the proportion of converted isolates in the southeastern regions was larger than 80%. In zones Ard-3, Ard-5, and LR-2, white isolates were found at a low frequency in spite of a high percentage of BCS (greater than 30%). Furthermore, there was no difference in the frequency of white isolates recovered in sites with or without BC in each region, except in Gard. These results suggest that the hypovirus released for BC had a limited spread in the field in France, as reported previously in the United States (32).

The restriction fragment length polymorphism markers developed by Alleman et al. (1) to study the diversity of CHV1

TABLE 4. Distribution of vegetative compatibility (VC) types of *Cryphonectria parasitica* in France

VC type	Converted by hypovirulent isolate ^a	Isolates of <i>C. parasitica</i>		White isolates	
		Number	%	Number	%
EU-2	2,022	622	55.8	185	65.8
EU-1	2,079	154	13.8	46	16.4
EU-5	2,079	79	7.1	23	8.2
EU-33	2,113	47	4.2	3	1.1
EU-65		44	4.0		
EU-12	2,100	29	2.6	5	1.8
EU-68	2,103	20	1.8	5	1.8
EU-14	2,022	16	1.4	6	2.1
EU-66	2,103	16	1.4		
EU-72		14	1.3		
EU-67		11	1.0	1	0.4
EU-74	2,113	9	0.8		
EU-3		8	0.7		
EU-13		6	0.5		
EU-24		6	0.5		
EU-11		4	0.4	2	0.7
EU-18		4	0.4		
EU-25		4	0.4	1	0.4
EU-38	2,113	3	0.3	1	0.4
EU-4		3	0.3	1	0.4
EU-30		2	0.2	1	0.4
EU-70		2	0.2		
EU-71		2	0.2		
EU-9	2,100	2	0.2		
EU-10		1	0.1		
EU-16		1	0.1		
EU-17	2,100	1	0.1		
EU-29		1	0.1		
EU-69	2,022	1	0.1	1	0.4
EU-73		1	0.1		
Total		1,113	100.0	281	100.0

^a Conversion to hypovirulence was tested with the hypovirulent strains listed in Table 1 and by the methods described in text.

TABLE 5. Allelic diversities at six *vic* loci in populations of *Cryphonectria parasitica*

Population	Number of isolates	<i>vic</i> 1	<i>vic</i> 2	<i>vic</i> 3	<i>vic</i> 4	<i>vic</i> 6	<i>vic</i> 7	Mean	Number of polymorphic loci ^a
Ardèche	292	0.085	0.201	0.027	0.007	0.066	0.098	0.081	2
Corsica	181	0.144	0.430	0	0.189	0.153	0.144	0.176	5
Dordogne	91	0.144	0.413	0.498	0.278	0.467	0.503	0.384	6
Gard	147	0.091	0.447	0.027	0.275	0.237	0.091	0.195	3
Lozère	171	0.121	0.500	0.057	0.306	0.180	0.090	0.209	4
Languedoc-Roussillon	114	0.068	0.415	0	0.190	0.101	0.132	0.151	4

^a A locus is considered polymorphic when the frequency of the less common allele is >0.05.

viruses could be used to track the spread of hypoviruses released for BC. Eight of our white isolates of *C. parasitica* from Corsica and Lozère, were studied by this method (1; unpublished data). Seven of them were infected by a hypovirus belonging to the Italian subtype, and one isolate was infected by a hypovirus related to one used for BC in France. This could be due to a lower conversion capacity of the BC agents than that of other hypovirulent strains (28,34). This finding confirms that the spread of hypovirulent isolates released for BC was low within sites and that the white isolates obtained were mainly due to a natural background level of hypovirulence.

In the coppice forests in Dordogne, hypovirulent isolates were never released, and the background level of hypovirulent isolates found was 5%. In the other regions the percentage of white isolates recovered in sites without BC may represent the natural background of hypovirulence where BC is applied. The natural background of hypovirulence was approximately 10% in Gard and Corsica, 20% in Ardèche and Languedoc-Roussillon, and 60% in Lozère. Similar levels of hypovirulence were reported for some areas in Switzerland and Italy where BC was not used (10,17,23,27). Thus, in France, as in other European countries, the spread of white isolates in the field appeared to be linked to factors other than BC. Confirmation of this theory will require further investigation.

The diversity of *C. parasitica* populations was the lowest in Corsica with only six VC types, a Shannon diversity index of 1.11, and a mean allelic diversity of 0.176. In contrast, high diversity was found in Dordogne, where we found 14 VC types, $H_s = 2.05$, and a mean allelic diversity of 0.384. The mean *vic*-allele diversity in Dordogne was higher than in southeastern populations and similar to the values reported for three populations of *C. parasitica* from the United States (36). The structure of *C. parasitica* populations in southeastern regions, where one VC type accounted for 50 to 70% of the isolates, is expected to strongly favor the transmission of hypoviruses compared to the population structure of Dordogne. At the zonal level, in Ard-5 and LR-2 the most frequent VC type gathered accounted for no more than 39% of the isolates, which may account for the lower spread of hypovirulent strains in these zones. Within Ardèche, the Shannon index was correlated with the percentage of white isolates, suggesting that the spread of hypovirulence depended on the diversity of the subpopulations. The nature of VC types present in *C. parasitica* may also influence the dissemination of CHV1 in *C. parasitica* populations, because *vic*-alleles have different effects on virus transmission (25,36). In Dordogne all *vic* genes were polymorphic, and allelic diversities at loci *vic* 3, *vic* 4, and *vic* 7 were higher than in the other populations.

Our results showed that VC-type diversity has decreased since 1981, i.e., 3 years after BC was used in the regions studied. The frequency of VC-type EU-2 increased significantly in all regions. In orchards the regular release of hypovirulent isolates of VC types EU-2 and EU-1 is likely to have increased the proportion of these VC types, due to the release of virus-free conidia (41). The spread of the CHV1 could also have resulted in changes in *C. parasitica* populations because of the inhibition of sexual reproduction of the fungus (3,47). However, more information about the mating type ratio and the frequency of fruiting bodies within populations are required to address the role of hypovirulence on the sexual behavior of *C. parasitica* in France. The occurrence of perithecia and the release of ascospores were reported in one orchard (21), but we have no indication of the outcrossing rate of *C. parasitica* in France (9,35,38). Our results showed that in southeastern populations, allele diversities of *vic* genes were different, and not all *vic* loci were polymorphic, suggesting nonpanmictic structure of the population, as reported in Italy (30). Sexual reproduction seems to have little influence on VC-type diversity in southeastern *C. parasitica* populations; the most common VC types differed by only one or two *vic* alleles.

The French (Dordogne) and Spanish western populations of *C. parasitica* (12) appeared different from the populations found in southeastern France, northern and southern Italy, Switzerland, eastern Spain, southeastern Europe, the Republic of Macedonia, Greece, and Bosnia (14,15,22). The differentiation pattern of *C. parasitica* populations in Europe suggests that gene flow between these three different regions of Europe is restricted, and existing populations may reflect founder effects. Differentiation of *C. parasitica* populations could be explained in France, as in eastern North America, by selection or genetic drift (37). However, analyses of *vic*-allele frequencies did not provide any evidence of selection acting against *vic* genes (36). On the other hand, European populations are relatively recent and they may not have reached their equilibrium, and it, therefore, is difficult to conclude on an eventual genetic drift. From a previous study, it was concluded that *C. parasitica* was introduced into Europe from Japan or North America (40), not from eastern China. However, the European populations used for this study did not represent all parts of Europe. Some introductions from China might have occurred in western Europe; Chinese chestnuts (*C. mollissima*) were introduced into France in the Pyrénées at the beginning of the century (43). Such a hypothesis is consistent with the distribution of the different subtypes of CHV1 and with the occurrence of new VC types found in our study in western France.

Our results show that six *vic* genes with two alleles are not enough to account for all the diversity of *C. parasitica* VC types in Europe. Higher diversity of *vic* genes has also been observed in China and the United States, where 131 and 67 VC types, respectively, have been reported (6,46). Although most of the American types are compatible with the 64 European testers, only 2 VC types from the 71 found in Japan were compatible with the European testers (13). A seventh *vic* gene or a third allele in one *vic* gene may occur in populations of *C. parasitica* in Asia and could have been introduced into western Europe. In France, additional VC types are expected in the Pyrénées, where isolates incompatible with the VC types were observed in the southeastern regions (19). The VC-type diversity of *C. parasitica* populations and their differentiation pattern indicate that adherence to quarantine regulations is essential not only to prevent the initiation of new blight canker foci, but also to avoid any increase in diversity in existing populations by introducing new *vic* alleles.

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