Chestnut Blight in Canada: Hypovirulence and Biological Control*

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ABSTRACT. Thirty-five isolates of *Cryphonectria parasitica* were collected from southern Ontario and characterized for culture morphology, growth rate, virulence on apple and live chestnut sprouts, and the presence of dsRNA. Five isolates were identified as hypovirulent but dsRNA was consistently isolated from only three (CCP-14, CCP-15, CCP-19). In several cases, virulent isolates contained detectable concentrations of dsRNA. Hypovirulent isolates CCP-28 and CCP-29 were isolated from the Amer tree, the best example of naturally occurring hypovirulence that has been found in Ontario to date. Double-stranded RNA was isolated only once from isolate CCP-28 and never from isolate CCP-29. There were 13 vegetative compatibility (v-c) groups identified among the 35 isolates from Ontario. All hypovirulent isolates from Ontario, and several known hypovirulent isolates from Europe and the United States, transferred hypovirulence and dsRNA to at least one v-c group. Hypovirulent isolates Ep 713, Ep 50 and GH 2 transferred hypovirulence and dsRNA to more than one v-c group. Northern hybridization analyses indicated that there were at least three unique dsRNAs associated with hypovirulence in

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Ontario. Hypovirulence-associated dsRNAs from isolate CCP-19 hybridized with dsRNA from isolate GH 2, indicating sequence relatedness between these hypovirulent isolates. Hypovirulent isolates (CCP-28, CCP-29) from the Amer tree were inoculated into virulent-type cankers and several cankers developed callus and cankers. The results establish that hypovirulent isolates of *C. parasitica* occur naturally in Ontario, and that inoculation with selected co-inoculum mixtures of hypovirulent isolates may represent a successful strategy for management of chestnut blight in Ontario.

The natural distribution of American chestnut *(Castanea dentata* [Marsh.] Borkh.) in Canada was primarily located within the Carolinian zone of southern Ontario, ranging from the western end of Lake Erie and the southern tip of Lake Huron eastward to the western end of Lake Ontario. This region represented the northwestern fringe of the natural range of American chestnut in eastern North America. Before chestnut blight, caused by *Cryphonectria parasitica* (Murr.) Barr, entered Ontario in the early 1920's, the chestnut population of this area was estimated at two million trees.

Between 1923 and the mid-1940's, the entire chestnut population of southern Ontario was devastated by blight. However, the species has continued to perpetuate within the Carolinian forests of Canada by regenerating from stumps and forming new sprouts. During the 1930's and 1940's, regenerating sprouts seldom attained a height of more than 3-4 m before being reinfected and killed by the pathogen. This cyclic pattern of regeneration and reinfection still continues but reinfection does not appear to be as prevalent as once observed. Population surveys for the distribution of American chestnut from 1979-1985 (1) and 1986-1991 (14, 15, 16) established that at least 96 sites of American chestnut were present in southern Ontario (Figure 1). Numerous trees within these sites appeared to have escaped and/or survived reinfection by the pathogen and have attained timber-tree stature (16). Approximately 50% of the trees that were 10 cm diameter at breast-height (dbh) were blight-free but the remaining trees have trunk and branch cankers.

There are several types of cankers among these infected trees. The most commonly observed types are rapidly developing cankers that completely girdle branches and trunks, and result in the death of terminal portions of the tree within a few months or 1 to 2 yr (Figure 2.2). There also are cankers of the typical healing type that have a restricted canker development and display annual callus growth at the edge of the cankers (Figure 2.1). Often, these trees develop new virulent-type infections within 3 to 10 yr and the main trunk of the tree dies. However, the most distinctive canker types observed within southern Ontario are the slow-developing, healing-type cankers that have been observed on a tree near Arner, Ontario. In 1966, this "Amer tree" was 30 cm dbh and was free from blight. By 1983, this tree had developed blight cankers on its lower trunk (Figure 2.3) but has continued to grow actively, and is now a much larger tree that shows little evidence of debilitation from blight infection (Figure 2.4).

The objectives of this study were to: assess chestnut sites within southern Ontario for healing cankers of chestnut blight; characterize hypovirulent isolates of *C. parasitica* associated with healing cankers; and, evaluate the potential for biological control of chestnut blight using hypovirulent isolates in southern Ontario. Preliminary reports of these studies have been presented (7, 17).

MATERIALS AND METHODS

Isolation and growth of *C. parasitica.* Isolates of *C. parasitica* were obtained by removing small samples of bark from the leading edge of chestnut blight cankers. Pure cultures were isolated by surface disinfection and/or baiting the fungus in apple fruit (cv. Granny Smith) and transferring the isolate to PDA or PDAmb. Isolates of *C. parasitica* from Europe and North America, and isolates representing known vegetative-compatibility groups from West Virginia, were supplied by W.L. MacDonald, West Virginia University, Morgantown, W.Va. These isolates were used as standards for comparisons of isolates from southern Ontario (Table 1).

Culture morphology. All isolates were evaluated for colony color, colony margin, radial growth and production of aerial mycelium and conidia. Typical culture morphology was represented by isolates that produced abundant aerial mycelium and conidia, had an undulate to entire colony margin, and grew to the edge of the petri-dish within 6-8 days. Atypical isolates produced sparse or appressed mycelium and/or reduced conidia production, and had an erratic colony margins. Radial growth in culture was measured under a 16-h photoperiod at 21-22 C for 7 days. The experiment was repeated three times and mean values were compared using cluster analysis (10).

Vegetative **compatibility and conversion capability.** The vegetative compatibility grouping for all isolates was determined following the technique of Anagnostakis (2) and Kohn et al. (12) except that red food coloring (Durkee-French Foods, Wayne, N.J.) was added to Difco PDAmb at a concentration of 1.0 ml dye/L of medium. To evaluate the transfer of dsRNA and associated hypovirulence, standard hypovirulent isolates (Ep 713, Ep 50, **JR 4,** GH 2), and hypovirulent isolates from Ontario (CCP-14, CCP-15, CCP-19, CCP-28, CCP-29), were paired with virulent Ontario isolates. All pairings were repeated at least twice and the colonies were incubated for 1 wk at 18-22 C in a 16-h photoperiod. Agar plugs were removed from the original virulent colony growth and transferred to a separate culture. If the culture grew slowly or had an altered morphology, the isolate was inoculated into apple alongside the original virulent and corresponding hypovirulent isolates. If the isolate had become hypovirulent (i.e. converted), the culture was assessed for the presence of dsRNA.

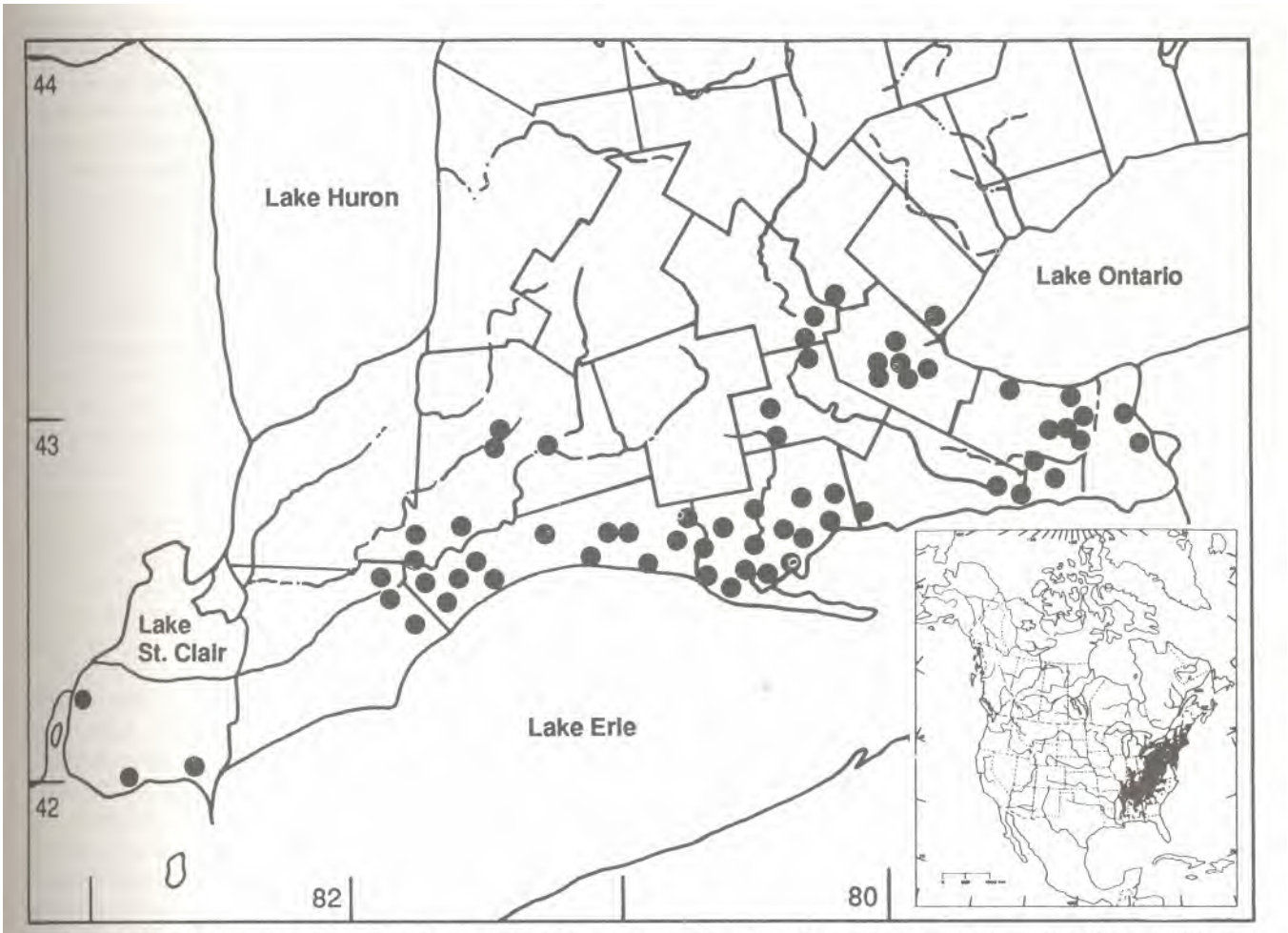

Figure 1. The distribution of stands and sites of American chestnut observed in surveys conducted in southern Ontario from 1979–1985 and 1986–1991. Inset shows the natural occurrence of American chestnut in eastern North America.

Virulence assays. The virulence of all isolates was tested on Golden Delicious apples according to the methods of Fulbright (8). The mean values for each isolate was compared using cluster analysis. The virulence of all isolates on American chestnut also was measured by inoculating live *C. dentata* sprouts. Six-mm-diameter holes were bored 4-6 mm into the chestnut sprouts at regular intervals (approximately 15 cm) in a spiral pattern around each stem. Each replication involved 2-3 sprouts of a single chestnut coppice. Sprouts ranged in diameter from 20 to 50 mm. A 6-mm-diameter agar plug was removed from the actively growing colony margin of each isolate and inserted into each hole. Sterile agar plugs were used as control treatments and inoculated chestnut tissues were covered with masking tape. Inoculated sprouts were allowed to develop for 6 wk. Lesion lengths after 6 wk were analyzed as an incomplete random block design with four replications and one observation per treatment. The experimental site was located near Wardsville, Ontario, approximately 50 km west of London, Ontario. Trials took place from June 13 to July 25, 1991, and from September 5 to October 17, 1991.

Inoculation of hypovirulent isolates into virulent cankers was conducted on young stems or sprouts (< 8-cm diameter) by transferring 5-mm-squares of PDA containing active fungus mycelium under small flaps of bark or, in older stems (> 8-cm diameter), a hand drill was used to bore 7-mm-diameter cavities through the bark to the cambium. Inoculation sites were sealed with plastic tape. The rate of canker development and the presence of callus formation was observed.

Purification of dsRNA. Isolation and purification of dsRNA from small samples (0.5 to 5.0 g) of tissue (method 1) of *C. parasitica* was conducted according to the method of Dodds & Bar-Joseph (5) and Hunst et al. (11), as described by Boland (4). Isolation and purification of dsRNA from larger samples (20 g) of tissue was conducted with a modified phenol extraction method (method 2). Sample preparations were chromatographed through a column containing 2.5 g of CF-11 cellulose powder saturated with STE-16.5% ethanol, followed by elution with 15 ml of 1 x STE. Contaminating single-stranded RNA and DNA were removed by T1 RNAse and DNAse 1 digestions, respectively, according to manufacturers instruction (Boehringer Mannheim, Laval, Que.). A second round of chromatography utilized 0.5 g of cellulose HL (InterSciences, Mississauga, Ont.) saturated with STE 16.5% ethanol, followed by elution in 1.5 ml STE. Re-

Figure 2. Virulent- and hypovirulent-type (healing) cankers of *Cryphonectria parasitica* on American chestnut in southern Ontario. (1) a healing canker representing naturally occurring hypovirulence; (2) a virulent-type canker in which the blight fungus invaded the main stem from an infected side branch; (3) the lower trunk of the Arner tree showing hypovirulent-type (healing) cankers in 1983; (4) the lower trunk of the Arner tree in 1991; (5) a virulent-type canker on trunk of American chestnut tree with inoculations by the Arner strain of *Cryphonectria parasitica* (CCP-28) around the perimeter of the canker; (6) canker shown in Figure 2.5, 17 months later. Note the bands of callus tissue around the perimeter of the canker.

covered nucleic acids were precipitated in 3 M Na acetate and 95% ethanol overnight at -20 C.

Northern blot analysis. Double-stranded RNA extracted from selected isolates of *C. parasitica* was electrophoresed in agarose gels, transferred to nylon membrane, and fixed according to the methods of Sambrook et al. (18). Random primed cDNA probes were prepared from 0.5-1.0µg of dsRNA that was denatured by boiling for 10 min. The reaction mixture consisted of 10µl of 5X reaction buffer, 3 µl of 100 mM DTT (both supplied with the enzyme; Gibco BRL, Burlington, Ont.), 5 ,u1100 O.D./ml DNA primers (Boehringer Mannheim, Laval, Que.), 2µl each of 15 mM dNTPs (Pharmacia, Baie d'Urfe, Que.), 50 alpha ^{32}P-Cl' (Amersham, Oakville, Ont.), 2µ1(400 units) superscript reverse transcriptase (Gibco BRL), and sterile H20 to a final volume of 50 ,u1. The reaction was incubated at 37 C for 90 min. After the reaction was complete, 250/4 of 10 mg/ml salmon sperm DNA (Pharmacia), 30µl of 7.5 M NH4 acetate and 750 ,u1 cold 95% ethanol was added to the tube. The nucleic acids were pelleted, washed in 95% ethanol and resuspended in 100 l 0.2 NaOH.

Membranes were pre-hybridized for 3-4 h at 43 C in a Hybaid hybridization oven (Bio/Can Scientific, Mississauga, Ont). The pre-hybridization solution contained 50% formamide, 0.25 M NaHPO4 (pH = 7.2), 1 M NaCL, ul mM EDTA, 100121 of salmon sperm DNA (10 mg/ml), 7.0% w/v SDS and water to a volume of 10 ml. Probe (50-100µl) was added to the pre-hybridization solution, and hybridization was performed overnight at 43 C. Membranes were washed twice in 2X SSC containing 0.1% SDS for 30 min at 37 C. Two more washes were performed for 30 min each at 37 C, using a solution of 25 mM NaHPO4 (pH = 7.2), 1 mM EDTA, and 0.1% SDS warmed to 50 C. Hybridization was visualized on Kodak X-Omat AR X-ray film (Sigma Chemical Co., St. Louis, Mo.) after exposing the film for 6 h to 10 days at -80 C with an intensifying screen.

RESULTS

Isolation of *C. parasitica.* Thirty-five isolates of C *parasitica* were collected from 15 sites in southern Ontario from 1985 to 1991 (Figure 1). Twenty-six isolates were collected from hypovirulent-type (healing) cankers, and nine isolates were collected from virulent-type cankers. Considerable variation in morphology, growth rate, cultural stability and virulence were observed among the isolates (Table 1).

Isolate morphology. Based on differences in colony color, colony margin, or production of aerial mycelium and conidia, or combinations of these characters, eight of 35 isolates exhibited atypical growth in culture (Table 1). Isolates CCP-28 and CCP-29, collected from the Amer tree. were unstable in terms of cultural growth and, after two or three colony mass transfers, these isolates occasionally showed reduced pigmentation and produced sparse, appressed mycelium, or stopped growing completely. Isolates CCP-1, CCP-2 and CCP-4 also were variable with respect to cultural characteristics, however, these isolates tended to grow as typical isolates more often than atypical. Isolates CCP-14, CCP-15 and CCP-19 were relatively stable and had smaller colony diameters when compared to standard virulent isolates. None of the isolates lacked pigment entirely as did hypovirulent isolates Ep 713, Ep 50, Ep 113, Euro 7 and virulent isolate Ep 32 C. Isolate CCP-19 was similar in culture morphology to hypovirulent isolate GH 2.

Cluster analysis of colony diameter measurements indicated that, among the 35 isolates from Ontario, there were three statistically different groups (Table 1). The two slowest-growing isolates were CCP-28 and CCP-29 (group a) but ten other slow-growing isolates composed group b. Both isolates from group a, and six of ten isolates from group b, also were categorized as atypical based on culture morphology. Group c included 23 isolates and represented isolates with the fastest growth rates. None of the group c isolates from Ontario were categorized as atypical based on cultural morphology. Standard virulent isolates were categorized in group c and standard hypovirulent isolates were categorized in groups a to c (Table 1).

Purification of dsRNA. All isolates of *C parasitica* were evaluated for the presence of dsRNA. All standard hypovirulent isolates and 13 of 35 Ontario isolates contained detectable concentrations of dsRNA. The amount of dsRNA recovered, and the number of bands visualized by electrophoresis, varied among the isolates (Table 1). Several virulent isolates tested positive for dsRNA on one or more instances.

Isolates CCP-1, CCP-2, CCP-14, CCP-15 and CCP-19 consistently revealed one band of dsRNA that was approximately 8-9 kb in size (Figure 3). Additional minor bands (< 1.0 kb) also were often observed. On one occasion, dsRNA was extracted from isolate CCP-28 but dsRNA was never recovered from CCP-29; both of these isolates were recovered from the Amer tree. D.W. Fulbright, Michigan State University, East Lansing, Mich., was unable to detect the presence of dsRNA in these isolates (D.W. Fulbright, personal communication).

Virulence assays. Mean separation analyses of lesion diameters from the apple virulence assay indicated that there were five statistically different groups. Standard hypovirulent isolates were included in groups a, b and c, and standard virulent isolates were clustered in groups c, d and e. Virulent-type lesions in apple appeared large, sunken, water-soaked and round to oval in shape.

Cluster analysis of lesion lengths from the chestnut sprout assay indicated that there were three statistically different groups (Table 1). Isolates CCP-14, CCP-15, CCP-19, CCP-28 and CCP-29 produced significantly smaller lesion lengths (group a) than all other Ontario isolates in both trials and were significantly similar to standard hypovirulent isolates. The remaining isolates were similar to standard virulent isolates. Data presented in Table 1 are the results from one of two field trials that were conducted. Results from the second trial were consistent with those from the first trial.

Table 1. Colony color, morphology, diameter on PDAmb after 7 days, presence of dsRNA and frequency of isolation, lesion diameter on apple after 14 days, and length of lesions on live *Castanea dentata* sprouts 6-wk-post-inoculation for isolates of *Cryphonectria parasitica.*

	Isolate	Morphology*	Diameter*	dsRNA**	Frequency of recovery**	Lesion Size (mm)† Apple	Chestnut	v-c Group‡
Canadian	CCP-1	Atypical	45.0b	+	Cons.	35.9d	37.8a	VC-1
Isolates	CCP-2	Atypical	42.9b	+	Cons.	19.5b	67.7c	VC-1
	CCP-4	Atypical	44.9b	–	–	24.7c	60.4b	VC-1
	CCP-14	Atypical	57.0b	+	Cons.	26.6c	43.8a	VC-7
	CCP-14	Atypical	62.3b	+	Cons.	30.0c	36.3a	VC-7
	CCP-19	Atypical	59.7b	+	Cons.	19.4b	36.4a	VC-2
	CCP-28	Atypical	28.4a	+++	1/6	14.0a	27.2a	VC-5
	CCP-29	Atypical	24.5a	–	–	28.1c	28.7a	VC-5
	CCP-3	Typical	73.5c	–	–	35.0d	58.7b	VC-1
	CCP-5	Typical	74.0c	–	–	35.8d	62.6b	VC-6
	CCP-6	Typical	68.3c	–	–	41.1e	70.9c	VC-8
	CCP-7	Typical	70.2c	–	–	38.8e	nt	VC-1
	CCP-8	Typical	73.1c	–	–	31.8c	62.1b	VC-6
	CCP-9	Typical	75.0c	+	2/5	31.3c	53.3b	VC-2
	CCP-10	Typical	72.0c	+	1/4	29.6c	55.4b	VC-6
	CCP-11	Typical	68.3c	+	1/5	36.4d	65.1c	VC-2
	CCP-12	Typical	72.4c	–	–	35.0d	66.8c	VC-4
	CCP-13	Typical	70.6c	–	–	33.9d	70.3c	VC-2
	CCP-16	Typical	56.7b	+	2/6	39.8e	62.3b	VC-9
	CCP-17	Typical	60.0b	+	2/5	26.8c	62.7b	VC-10
	CCP-18	Typical	73.9c	–	–	37.4d	64.1b	VC-4
	CCP-20	Typical	72.1c	–	–	35.0d	68.3c	VC-11
	CCP-21	Typical	71.5c	–	–	33.2c	59.7c	VC-1
	CCP-22	Typical	77.7c	+	1/5	38.6e	61.2b	VC-12
	CCP-23	Typical	63.7b	–	–	31.7c	54.2b	VC-4
	CCP-24	Typical	55.3b	–	–	35.8d	60.9b	VC-13
	CCP-25	Typical	71.9c	–	–	41.0e	61.9b	VC-3
	CCP-26	Typical	71.8c	–	–	39.7e	62.9b	VC-3
	CCP-27	Typical	65.6c	–	–	33.1c	66.6c	VC-1
	CCP-30	Typical	75.2c	–	–	33.3c	75.5c	VC-3
	CCP-31	Typical	70.1c	–	–	34.8d	60.1b	VC-3
	CCP-32	Typical	71.2c	+	1/4	48.9e	60.9b	VC-3
	CCP-33	Typical	74.1c	–	–	46.5e	63.0b	VC-3
	CCP-34	Typical	70.3c	–	–	37.8d	67.4c	VC-2
	CCP-35	Typical	76.0c	–	–	39.4e	68.6c	VC-2
Standard	EP 50	Atypical	59.2b	+	–	15.9b	nt	–
Hypovirulent	EP 113	Atypical	54.1b	+	–	12.7a	nt	–
Isolates	EP 713	Atypical	68.9c	+	–	17.8b	36.8a	–
	Euro 7	Atypical	81.3c	+	–	30.1c	nt	–
	GH 2	Atypical	55.3b	+	–	17.7b	34.5a	–
	JR 4	Atypical	35.9a	+	–	10.1a	nt	–
Standard	5-9-1B	Atypical	77.0c	–	–	45.0e	nt	–
Virulent	CL-1-16	Typical	77.7c	–	–	24.5c	56.8b	–
Isolates	Ep 32 C	Atypical	83.2c	–	–	39.1e	nt	–
	Ep 155	Typical	67.7c	–	–	35.7d	69.7c	–
	Ep 408	Typical	77.6c	–	–	34.4d	nt	–
	Ep 523	Typical	80.2c	–	–	43.9e	nt	–

*Colony morphology and diameter of isolates determined after 7-days growth on PDAmb. Isolates were categorized as typical or atypical based on comparisons to standard virulent or hypovirulent isolates, respectively. Measurements of colony diameters are means of four replications. Means within a column followed by the same letter are not significantly different according to cluster analysis (P = 0.05).

**The number of + signs = the number of bands commonly observed by agarose gel electrophoresis. Where dsRNA was not recovered consistently, a ratio of success (e.g. 2/5) is presented.

†Measurements of lesion diameters (apple) and lesion lengths (chestnut) are means of four replications. Means within a column followed by the same letter are not significantly different according to cluster analysis (P = 0.05).

‡Vegetative compatibility groups of isolates of *C. parasitica* collected from southern Ontario from 1979 to 1991.

nt = Isolate not tested.

Isolates CCP-14, CCP-15, CCP-19, CCP-28, CCP-29 and the standard hypovirulent isolates failed to produce notable mycelial fans beneath the bark of hypovirulent-type cankers. Isolates CCP-28 and CCP-29 consistently produced swollen split cankers, whereas CCP-14, CCP-15, GH 2 and Ep 713 occasionally formed similar cankers.

Vegetative compatibility and conversion capability. Thirteen vegetative compatibility (v-c) groups were distinguished among the 35 isolates from southern Ontario (Table 1). Groups VC-1, VC-2 and VC-3 each contained 7, 6 and 6 isolates, respectively; groups VC-4 and VC-6 each contained 3 isolates; groups VC-5 and VC-7 each contained 2 isolates; and the remaining groups each contained one isolate. The conversion capability of individual hypovirulent isolates was established among the Ontario v-c groups *in vitro*. Ontario v-c groups VC-1, VC-4 and VC-11, and individual isolates from groups VC-2 (CCP-35) and VC-3 (CCP-30), were converted by Michigan hypovirulent isolate, GH 2. European hypovirulent isolates, Ep 713 and Ep 50, were each able to transform groups VC-6 and VC-9. Isolate CCP-20 also was converted to hypovirulence by Ep 50. One of the Ontario hypovirulent isolates, CCP-19, converted all members of group VC-2. Ontario hypovirulent isolates CCP-28 and CCP-29 each converted virulent isolate CCP-17, which represented group VC-10. Hypovirulent isolates CCP-14, CCP-15 and JR 4 did not convert any virulent Ontario isolates. Except for isolate CCP-17, which failed to grow after the conversion event, the dsRNA banding pattern extracted from the converted isolates matched the dsRNA pattern of the donor hypovirulent isolates.

Figure 3. Agarose gel banding patterns of dsRNAs associated with *Cryphonectria parasitica*. λ is a molecular weight marker of Hind III digested Lambda DNA. Lanes 1 and 2 contain dsRNAs from standard hypovirulent isolates Ep 713 and GH 2, respectively. Lane 3 contains dsRNA that was typically extracted from isolates CCP-14 and CCP-15 (CCP-14 presented). Lane 4 contains dsRNA that was similar in size to dsRNA in lane 3, and was typical of dsRNA extracted from isolates CCP-1 and CCP-2 (CCP-1 presented). Lane 6 contains dsRNA extracted from isolate CCP-19.

Northern blot analysis. Northern blot hybridizations were conducted to determine the genetic relatedness of dsRNA in isolates collected from southern Ontario with hypovirulent isolates from other locations. Preliminary results indicated that dsRNA from CCP-14 hybridized with minor bands from isolates Ep 713 and CCP-19 but not with larger hypovirulence-associated bands. Radiolabelled probe prepared from dsRNA in isolate GH 2 hybridized with dsRNA from isolate CCP-19. Hybridization also occurred with some minor bands in this analysis. None of the other dsRNA bands showed a clear hybridization signal with dsRNA from isolates CCP-15 or GH 2. The hybridization signals that were observed with the minor bands in some hybridizations may have been due to contaminating cellular RNAs in the purified dsRNA preparations.

Naturally occurring hypovirulence in the Amer tree. A unique type of cankering has been observed in an American chestnut tree located at Arner, Ontario. Annual inspections from 1966 to 1972 showed the Amer tree to be blight-free. For eleven years after 1972, no inspections were made. In the summer of 1983, several cankers with somewhat ill-defined borders (Figure 2.3) were observed on the lower 2.4 m of the trunk. These cankers ranged up to 30 cm in diameter. The removal of loosened outer bark from two of the cankers exposed abundant callusing and phloem regeneration beneath. A cankered dead stem, 8 cm dbh, had originated from the base of the trunk. A smaller living sprout had cankers on its branches that killed the terminal portions.

Frequent examinations of the Amer tree since 1983 have shown that the superficial trunk cankers became less discrete as they slowly increased in diameter. The callus tissue over the cankers was somewhat uneven with a rough surface. A pale, creamy orange tint was reflected from the canker surface when moist. By 1992, the outline of the cankers had become less distinct (Figure 2.4) and the increased growth of callus on the lower trunk had given the bole a slightly tapered appearance, unlike those of large blight-free chestnut tree trunks. Blight has not spread to the upper trunk or into the crown. Sprouts have emerged from the base of the Amer tree, and all of these sprouts have developed cankers in their branches, killing the terminal growth. To date, perithecia have not been observed on the Amer tree or on any *C. dentata* stems inoculated with isolates recovered from the Amer tree. Careful, shallow excavation at one side of the base of the Arner tree has revealed that the tree originally arose from a rotting chestnut stump. The Arner tree continues to be a canopy-maker with a present height of approximately 24 m and a dbh of 71 cm.

Inoculation of virulent-type cankers with hypovirulent isolates. Because the Amer isolates of *C. parasitica* have been most effective in producing healing cankers in nature, these isolates (CCP-28 and CCP-29) were used in field inoculations. Isolates showing medium growth rates in culture were used because the slowest growing cultures frequently fail to produce conspicuous infections. Inoculations of virulent-type cankers were conducted by inserting inoculum into cavities in the bark extending to the cambium around the perimeter of the canker. Inoculations were spaced at 5-cm intervals and 2 to 3 cm beyond the advancing canker margins (Figure 2.5). Inoculations were made during May and June, 1991.

Inoculations placed under a small flap of bark in young stems of *C. dentata* that were < 4 cm dbh usually resulted in girdling and death of the distal portions. In stems > 5 cm dbh, the fungus stimulated callus formation and resulted in considerable hypertrophy at the canker site, giving rise to healing cankers (Figure 2.5). The amount of necrosis at the immediate site of the inoculation has been variable.

Stems > 15 cm dbh with virulent-type cankers that were inoculated around their perimeter with the Amer isolates have shown arrest of virulent-type cankers. Changes in bark color around the sites of inoculation accompanied by some hypertrophy indicate that callusing was initiated. Bark covering the virulent-type canker showed prominent cracking within 8 to 10 mo. Excision of cracked and loosened bark over the canker at 12 mo after inoculation showed a prominent bank of callus tissue at the canker's edge (Figure 2.6). Isolations from near the edge of the canker at 15 and 18 mo (Figure 2.6) after inoculation with the hypovirulent isolate have yielded both virulent and hypovirulent isolates. Experiments are in progress to determine whether virulent isolates were converted to hypovirulent.

DISCUSSION

Of the 35 isolates of *C. parasitica* that were sampled from populations of American chestnut in Ontario in this study, most isolates (77%) were similar to standard virulent isolates collected from other locations. In previous studies, isolates from 15 additional locations also were characterized as virulent, based on colony morphology and virulence in apple. Based on the collective data from cultural morphologies, the presence of dsRNA, and virulence assays in apple and fruit, five of the isolates from southern Ontario (CCP-14, CCP-15, CCP-19, CCP-28 and CCP-29) were considered to be hypovirulent. All of these isolates were reduced in virulence and possessed at least one of the characteristics commonly associated with hypovirulence. Three of these isolates appeared particularly promising as biological control agents. Isolate CCP-19 was significantly reduced in virulence and was similar to the standard hypovirulent isolate, GH 2. However, compared to isolate GH 2, isolate CCP-19 produced approximately ten times more conidia per plate. The increased sporulation displayed by this isolate has implications for the biological control of chestnut blight. Such an isolate could provide a more prevalent source of hypovirulent inoculum for interaction with virulent inoculum. Isolates CCP-28 and CCP-29, recovered from the Amer tree, were considered to be hypovirulent despite the variable presence of dsRNA. Failure to extract dsRNA from these isolates does not negate their value as biological control agents (3, 9). The cytoplasmic element responsible for hypovirulence in CCP-28 and CCP-29 is transmissible and, therefore, valuable as a potential biological control.

In several cases, apparently normal, virulent isolates contained detectable concentrations of dsRNA. In all such cases, the dsRNA appeared as a large (approximately 9-10 kb) weak band when visualized by agarose electrophoresis. Up to one-third of the isolates tested from West Virginia also possessed a small amount of dsRNA not associated with altered virulence (6). It is possible that two classes of dsRNA may exist in Ontario populations of *C. parasitica;* hypovirulence-associated dsRNA and cryptic dsRNA. Hybridization analyses between these dsRNAs should establish their sequence relatedness.

All isolates produced lesions when inoculated into apples, and lesion diameters in apple were highly correlated with virulence of the same isolates in chestnut sprouts ($r = 0.90$, $P = 0.05$). There was a moderate correlation ($r = 0.69$, $P = 0.05$) between growth on PDAmb and virulence in chestnut.

The ability of hypovirulence to spread in natural populations of *C. parasitica* is affected by the diversity of v-c groups present. Thirteen v-c groups were detected among the 35 isolates evaluated from southern Ontario, and there was no apparent distribution pattern of the v-c groups among the sampling sites examined. While this sample size is small, the large number of v-c groups that were identified indicates a diverse population within southern Ontario.

Transmission of dsRNA and hypovirulence was demonstrated by Ontario hypovirulent isolates CCP-19, CCP-28 and CCP-29. Hypovirulent isolates CCP-14 and CCP-15 did not convert any virulent Ontario isolates. This is probably because these isolates were the only members of v-c group, VC-7. Similarly, hypovirulent isolate JR 4 did not convert any virulent Ontario isolates. Hypovirulent isolates GH 2, Ep 713 and Ep 50 were each able to convert more than one v-c group of virulent isolates from Ontario but isolate CCP-19 only transmitted hypovirulence and dsRNA to isolates from the same v-c group.

Mixtures of hypovirulent isolates that are capable of transferring dsRNA among several v-c groups may provide adequate control in a diverse population of virulent *C. parasitica* (13). Based on conversion experiments conducted in culture, 80% of the virulent isolates from Ontario were converted by the hypovirulent isolates used in this study. In particular, hypovirulent isolate GH 2 converted 13 of 30 (43%) virulent Ontario isolates tested and hypovirulent isolate CCP-19 converted six Ontario iso-

later (20%). If isolates GH 2 and CCP-19 were used as a co-inoculum treatment, up to 63% of the virulent Ontario isolates could be converted, representing just under one-half of the v-c groups. European hypovirulent isolates Ep 713 and Ep 50 consistently converted four isolates (13%) representing two v-c groups. Inclusion of several hypovirulent isolates in a co-inoculum treatment may increase conversion flexibility in mixed v-c populations of C. *parasitica*.

Northern blot analyses of dsRNAs were conducted to identify dsRNAs that were common among the hypovirulent isolates from Ontario and elsewhere. Preliminary results indicated that dsRNAs in isolates GH 2 and CCP-19 were genetically related. These isolates also were similar in cultural morphology. Homology between dsRNA from GH 2 and CCP-19 may explain some of the behavioral similarities between these isolates. The relatedness of dsRNA from other hypovirulent isolates from Ontario has not been determined but, based on preliminary experiments, there appears to be at least two additional unique dsRNAs associated with these hypovirulent isolates.

The best example of naturally occurring hypovirulence that has been found in Ontario to date is in the "Amer" tree (isolates CCP-28 and CCP-29). The blight in the Amer tree appears to be unique. The existing cankers have not perceptibly jeopardized the overall growth of the tree during the last decade. However, canker activity has been obvious and isolations of *C. parasitica* have been readily made. As in the Amer tree, other *C. dentata* trees (> 20 cm dbh) that were inoculated with isolates CCP-28 and CCP-29 have produced callus growth.

In culture, the growth rates of isolates from the Amer tree were variable but often greatly reduced. On several occasions these isolates became so debilitated that they failed to grow on agar. On PDA and other culture media, the Amer isolates vary appreciably in the production of pycnidia initials and hyphal conidia. Sporulation does not always bear a direct relationship to mycelial production. Occasionally, isolates CCP-28 and CCP-29, after several mycelial transfers, produced a minimum of mycelial growth but an abundance of hyphal conidia.

The increasing number of timber quality chestnut trees in southwestern Ontario signifies a recovery of American chestnut in the province (16). This recovery may be attributed particularly to two factors. First, there has been a steady decrease in the amount of inoculum of *C. parasitica* due to the rotting of old chestnut stumps with the *resulting* inability of these sites to regenerate and produce new sprouts. Second, an increasing number of blighted trees contain hypovirulent isolates of the pathogen that are associated with healing cankers. This naturally occurring development of hypovirulence appears to be associated with an increased number of trees that are reaching reproductive age. If hypovirulence in the Amer isolates can be transferred by hyphal anastomosis to virulent strains of the pathogen in field environments, the Amer strain may serve as an important biological control agent. Within a year or two, field experiments in progress should establish whether the Amer isolates offer such promise. Meanwhile laboratory studies are concentrating on determining the mechanisms of hypovirulence in these, and other isolates of the pathogen.

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