Investigations on Hypovirulence in Chestnut Blight in Croatia

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ABSTRACT. Chestnut blight, caused by the fungus Cryphonectria *parasitica* has spread throughout the natural range of chestnut in Croatia. Hypovirulence is appearing spontaneously, and is manifested in healing cankers and superficial necrosis. The review of investigations on chestnut blight hypovirulence, and results of initial experiments for disease control by hypovirulent strains, are presented and discussed.

European sweet chestnut *(Castanea saliva Mill.) is* an indigenous forest tree species in Yugoslavia. Because of its specific ecological requirements for a moderately warm, humid climate, and rich acidic soil, chestnut is located only on certain sites, and covers about 70,000 ha. In pure stands it grows as coppice. In mixed stands it is often part of the canopy that also includes *Quercus sessiliflora* (Salisb.) Smith, *Quercus pubescens* (Thuill.) Lamk., *Carpinus betulus* L. and *Fagus silvatica* L. Sweet chestnut is an important forest tree because of its value as timber, as raw material for tannin extraction, and its edible fruit. In some areas it is an important source of income for the local population.

Chestnut blight, caused by the fungus *Cryphonectria parasitica* (Murr.) Barr, was first reported in 1950 in Nova Gorica, a district near the Slovenian-Italian border (8). The disease spread rapidly throughout Slovenia, Croatia, into Bosnia and Hercegovina, and as far as the southern part of the country into Macedonia. In spite of strict quarantine regulations to halt its spread, after 28 yr, all chestnut forests were affected by *C. parasitica.* The infection intensity varied from single trees in mixed stands with oak, through group infections, to extensive infections in pure chestnut coppice stands. Until now, we have found only two isolated localities without chestnut blight: Srebrenica in Bosnia, and Koprivnica in Croatia.

During the first years of *C. parasitica* attack the disease was manifested in a very aggressive form, and many chestnut forests had to be cut. Some of them were reforested with other forest tree species, and others regenerated from stumps as chestnut coppice forests. Many new sprouts and young trees were again affected with the disease, but on some localities the cankers showed slower, abnormal expansion.

Recently, the problem of chestnut blight has again attracted attention. New developments in chestnut blight in other countries, and the discovery of atypical disease symptoms and hypovirulent *C. parasitica* strains, have prompted us to investigate more thoroughly the characteristics of the fungus and the new phenomenon of hypovirulence in chestnut forests in Yugoslavia.

NEW INVESTIGATIONS ON CHESTNUT BLIGHT IN CROATIA

About 10 yr ago, besides the standard symptom of bark necrosis, non-standard symptoms were observed in some chestnut forests (5, 6, 9). In some chestnut coppice stands the tree did not die at the rate the intensity of infection would indicate. Several trees with more than one canker (1-6) along the stem were still alive. On most of those trees, strong callus tissue developed around the cankers. This tended to grow stronger, and the size of wounds gradually decreased (7). Also observed was the superficial bark necrosis that is especially visible on branches and stems in the smooth bark stage. The following three types of cankers formed occur separately, although two or even three may be found on the same tree. Their traits are briefly given in three categories:

1.Active cankers (AC): Infection rapidly extends to the vascular cambium. The bark first becomes sunken, and then changes color and splits, and finally the wound opens. Typical are the dirty yellow mycelium fans. Fructifications are yellow-brown and abundant. Leaves on branches or stems wilt conspicuously. Water sprouts appear below the infected spot.

2. Callused cankers (CC): Infection extends to the vascular cambium. Around the colonization, callus tissue is formed, with the tendency to close over the exposed wood. Fructifications are sparse to nonexistent. Water sprouts are rare.

3. *Superficial necrosis* (SN): Infection progresses slowly and does not extend to the vascular cambium. Fructifications are sparse to nonexistent and hypertrophy is common. No water sprouts form below the infection spot.

Isolates derived from these cankers yielded morphologically different *C. parasitica* colonies, which varied from strongly pigmented to quite white. The fungal isolates were categorized according to their traits:

Normal cultures (N) were orange pigmented and produced many small pycnidia, usually arranged in concentric rings. They were isolated from active cankers and represented 39% of the 59 isolates tested.

White cultures (B) were isolated mostly from superficial necroses, callused cankers, and in several cases from cankers assumed to be active cankers, such as isolates 967/37; 1131/56; 1132/57 and 1133/58. Cultural characteristics include radially symmetrical growth and white mycelium with abundant aerial mycelium. White cultures formed

few, large pycnidia after long incubation periods; in some cases only a single pycnidium was produced. Such cultures represented 17% of the collection.

Cultures with *intermediary characteristics* (I) varied in pigmentation from pale yellow to nearly orange. Pycnidia were usually large and appeared less frequently than in normal cultures. Their size, number and arrangement in cultures varied from one strain to another. Further abnormalities in these cultures included irregular growth, or growth rate of mycelium. Intermediary cultures were isolated in large number, and represented 46% of the collection.

In laboratory culture, some white and poorly pigmented isolates of *C. parasitica* were found to be unstable, in terms of culture morphology and pathogenicity. Some of them developed segments or sectors of increased pigmentation. As pigmentation changed from pale yellow to orange, hypovirulent traits disappeared and virulence increased, as determined by pathogenicity tests.

PATHOGENICITY TESTS FOR *CRYPHONECTRIA PARASITICA* ISOLATES

Pathogenicity tests were performed *in vitro* and *in vivo* for 58 isolates of *C. parasitica* derived from all categories of canker formations, according to Elliston (4). In both tests, the average canker areas obtained from four inoculations showed a continuous decrease in the pathogenicity from highest to lowest values for developed cankers (Table 1 and Figures 1 and 2). It is evident that the lowest values for canker area belong mostly to white isolates, and the highest values mostly to those designated "N" orange isolates. This agrees with previous work on hypovirulent and virulent strains. There are, however, significant deviations; among white isolates with low canker areas, there are orange isolates derived from active cankers, i.e. isolates 26, 58, 41, etc. Conversely, isolate 11, a white isolate, formed a large canker. Analyzing the pathogenicity data, the question arises whether the pigmentation evaluation was correct, since some isolates appeared to have variable characteristics, and to be more or less virulent than expected. It would be difficult to distinguish virulent from hypovirulent strains on this basis. In any case, pathogenicity tests do not provide sufficient evidence on virulence of individual isolates, and further tests are required.

TESTING VEGETATIVE COMPATIBILITY OF *CRYPHONECTRIA PARASITICA* STRAINS

Our intention was to find out to what extent compatible strains exist in Croatia, and what interaction occurs between the hypovirulent and virulent strains. The vegetative compatibility (v-c) trials were conducted with 59 representative isolates from various sites in Croatia and Slovenia and included isolates from all three canker categories. Vegetative compatibility testing was conducted according to Anagnostakis (1). Tested isolates, numbered 1-59, are listed in Table 1. Origin and the canker type are given for each canker type. Symbols denote the

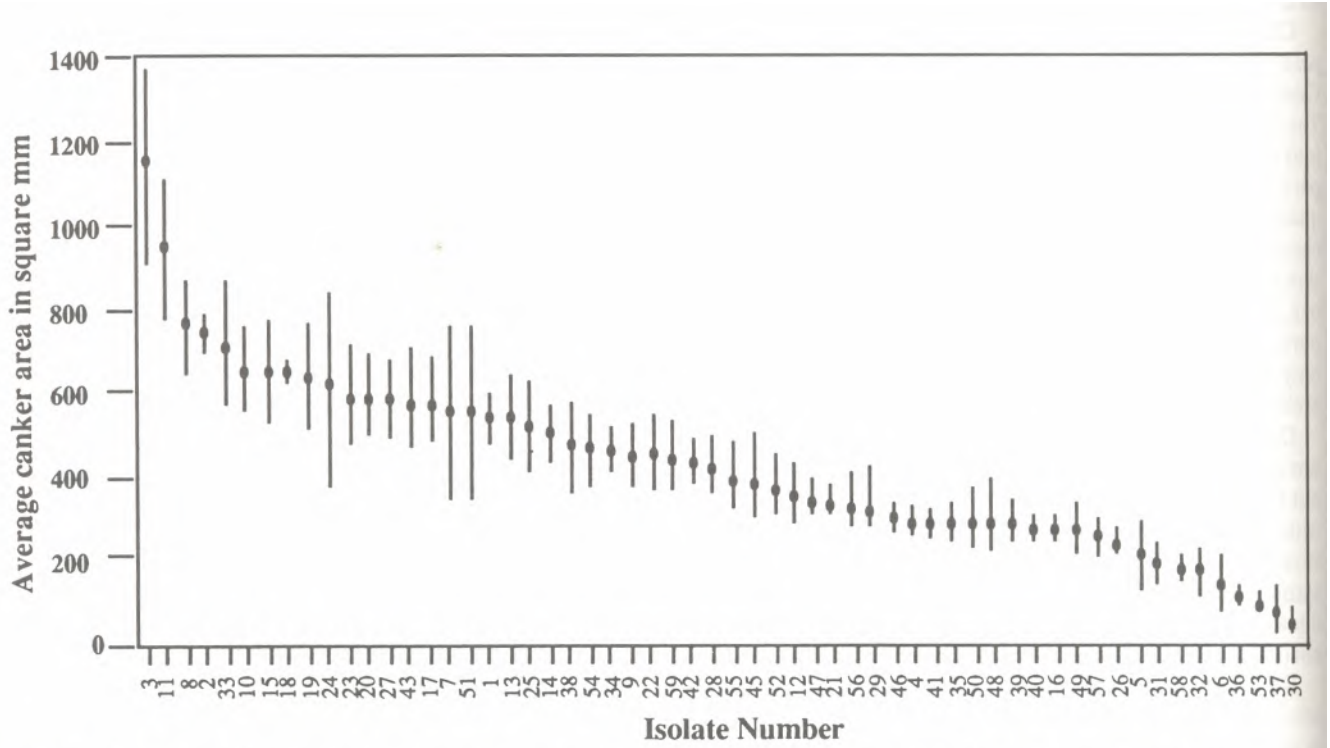

Figure 1. Pathogenicity of 58 *Cryphonectria parasitica* isolates on excised chestnut stems 5 wk after inoculation (arranged according to decreasing canker area).

Table 1. Characteristics of 59 *Cryphonectria parasitica* isolates, vegetative compatibility tests and pathogenicity *in vitro*.

Number in test	Isolate number	Origin	Source	Cultures Morphology*	Cultures Growth in mm	Number of compatible pairs	Pathogenicity *in vitro* Canker mm^2	Pathogenicity *in vitro* area S.D.	Pycnidia**
1	694	Glina	CC	I	82	18	537	50.4	–
2	797	Nova Gorica	CC	I	80	11	744	30.7	+
3	727/1	Petrova Gora	CC	N	78	9	1127	239.7	+
4	727/2	" "	CC	I	75	8	332	43.6	+
5	780	Osredek, Krsko	CC	B	86	21	205	74.3	–
6	793	Glina	CC	B	67	8	139	58.2	–
7	822/1	Petrinja	CC	I	87	13	550	193.2	–
8	822/2	"	CC	N	57	17	772	109.0	–
9	823	"	CC	I	84	6	459	29.7	+
10	832	Petrova Gora	AC	N	76	6	638	103.8	+
11	833	Ucka	SN	B	83	6	944	158.1	+
12	835	"	SN	I	78	3	373	49.6	++
13	836	"	SN	I	89	9	633	23.0	+
14	838	"	AC	N	75	2	507	64.0	+
15	855	Zumberak	AC	I	88	3	636	127.0	+
16	888	Petrova Gora	CC	B	52	9	277	23.2	–
17	915	Koprivnica	AC	N	75	6	566	82.1	++
18	915/2	"	AC	N	87	6	519	67.2	++
19	985/1	Petrinja	SN	I	80	25	617	112.7	++
20	994	Krizevci	AC	N	82	5	586	83.3	+
21	996	Buje	CC	I	81	8	359	14.9	+
22	997	"	CC	I	74	2	449	84.7	++
23	1002	Glina	CC	I	84	13	593	124.7	++
24	1003	"	SN	I	53	10	609	223.6	+
25	1004	"	SN	I	50	16	519	94.8	+
26	745	Rude, Samobor	AC	N	86	10	229	26.2	+
27	780/d	Osredek, Krsko	CC	I	70	2	579	83.7	++
28	781	Sevnica	AC	N	67	4	431	61.3	+
29	821/a	Petrinja	CC	I	89	1	358	58.9	++
30	824/a	Glina	SN	B	78	20	48	14.9	+
31	825	Glina	CC	N	84	5	192	39.6	+
32	827	"	SN	I	78	6	171	40.2	+
33	901	Koprivnica	AC	N	82	6	704	166.8	+
34	964	Petrinja	CC	I	90	3	436	49.1	+
35	965	"	AC	N	87	3	316	43.3	+
36	966	Petrinja	CC	I	89	9	104	8.4	–
37	967	"	AC	B	90	15	81	45.8	–
38	968/1	"	AC	I	86	3	471	103.5	++
39	969/2	"	AC	I	90	2	300	50.8	+
40	969	"	CC	I	82	7	277	21.9	+
41	970/1	Petrinja	AC	N	80	3	319	28.0	+
42	970/2	"	AC	N	78	8	441	45.3	+
43	971	"	AC	N	79	1	575	133.8	++
44	972	"	CC	I	45	0	not tested		
45	973	"	CC	N	81	4	401	88.9	++

Number in test	Isolate number	Origin	Source	Cultures Morphology*	Cultures Growth in mm	Number of compatible pairs	Pathogenicity *in vitro* Canker area mm^2	Pathogenicity *in vitro* S.D.	Pathogenicity *in vitro* Pycnidia**
46	974/1	Petrinja	AC	N	–	4	347	28.5	++
47	974/2	"	AC	N	81	3	370	23.9	+
48	975	"	CC	I	85	7	300	91.7	++
49	976	"	SN	I	80	6	273	62.8	++
50	977	"	SN	I	88	7	303	65.0	+
51	983	Petrinja	AC	I	80	3	547	182.0	++
52	985/1	"	CC	I	90	5	393	45.6	++
53	985/2	"	SN	B	89	14	88	4.0	+
54	987	"	AC	N	78	2	472	87.9	++
55	804	Buje	AC	N	77	5	409	66.3	+
56	1131	Petrova Gora	AC	B	–	22	358	55.4	++
57	1132	" "	AC	B	–	23	242	45.3	+
58	1133	" "	AC	B	–	9	178	20.6	+
59	1134	" "	AC	I	–	22	447	79.6	++

* Morphological characteristics of cultures 10 days after inoculation at room conditions:
N—normal cultures, the wild type of orange pigmentation, produces abundant small pycnidia; B—white cultures with rare large pycnidia; I—cultures with intermediary characteristics

**No pycnidia (–); Rare pycnidia (+); numerous pycnidia (++)

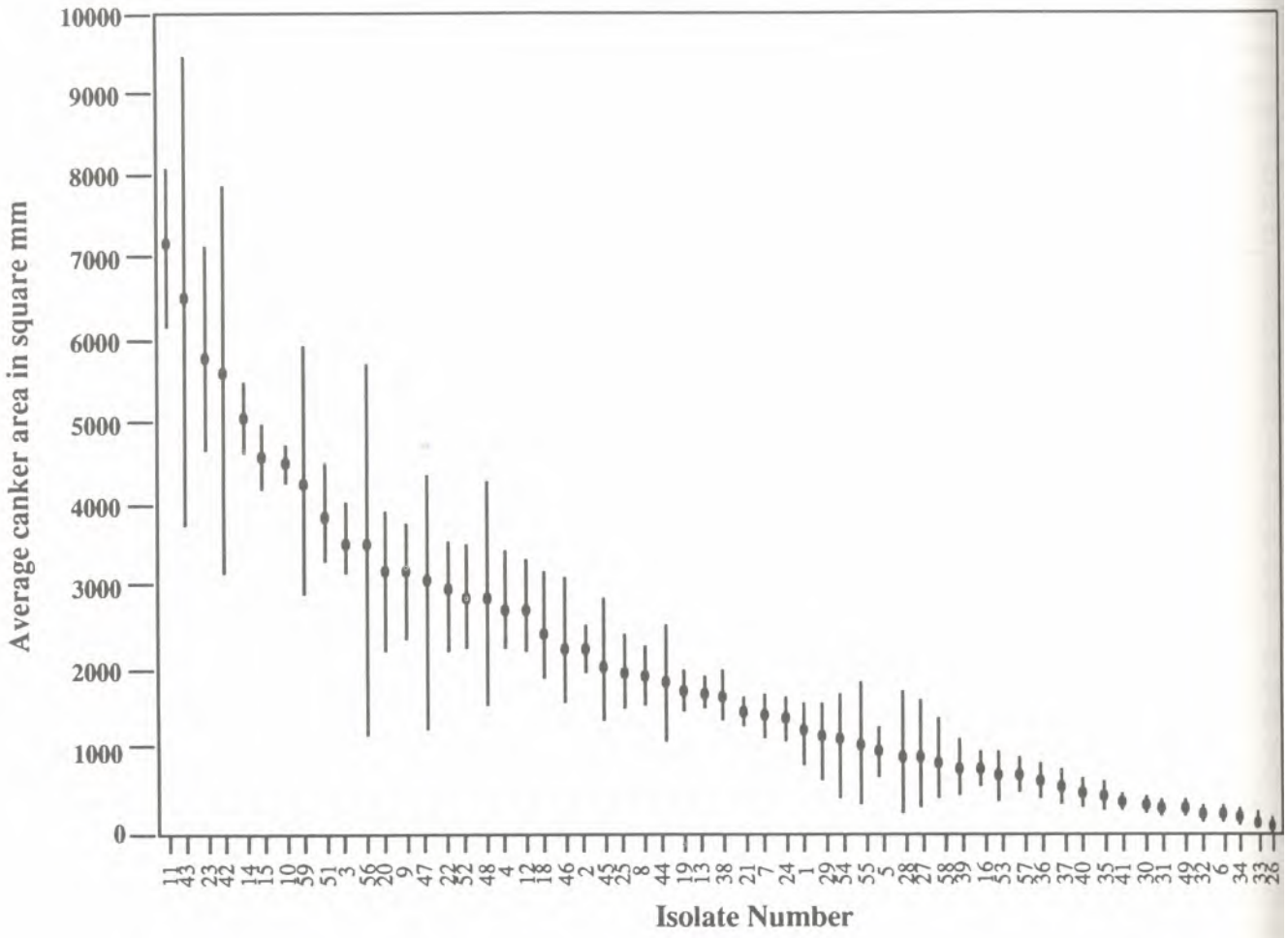

Figure 2. Pathogenicity of 57 *Cryphonectria parasitica* isolates on chestnut trees in the forest, 16 wk after inoculation (arranged in decreasing canker area).

culture pigmentation and the number of compatible pairs formed by each strain in the v-c test are listed. The analysis of data obtained from this test showed that isolates of *C. parasitica* are more often incompatible than compatible. In our collection, the total incidence of compatibility was 14%. The highest percentage of compatible pairs was obtained by pairing isolates from Petrova Gora (37-43%) with the isolates from the Petrinja region. Petrova Gora and Petrinja are areas with the highest intensity of infection. It also is significant that compatible virulent and hypovirulent isolates were obtained from distant provenances (Istria, Krizevci, Slovenija, Petrova Gora).

IDENTIFICATION OF DOUBLE-STRANDED RNA

The hypovirulent strains contain cytoplasmic double-stranded RNA, that is correlated with reduced virulence and sporulation. The purpose of this research was to determine whether dsRNA is present in isolates previously shown to be less pathogenic and exhibit morphological characteristics of hypovirulent strains.

The analysis for dsRNA of 14 of our isolates was carried out at the Federal Institute of Forestay Research in Birmensdorf, Switzerland, headed by Ursula Heiniger. Isolation, purification and determination of dsRNA by gel-electrophoresis were done according to modified methods of Day et al. (2) and Dodds (3). DsRNA was detected in 9 out of 14 tested isolates. Test results are given in Table 2.

The first group of five isolates in Table 2 produced cankers, relatively large in size, in all tests. Only isolate 977 deviated; it was less pathogenic, with intermediate culture characteristics. Two other groups are assumed to be hypovirulent based on their morphological characters and pathogenicity, and these were shown to contain dsRNA (Table 2).

Table 2. Double-stranded RNA in *Cryphonectria parasitica* isolates.

Number	Isolates Origin	Source	Culture Char.*	dsRNA
804	Istra, Buje	AC	N	–
823	Petrinja	CC	N	–
833/II	Istra, Ucka	CC	I	–
855	Zumberak	AC	N	
977	Petrinja	SN	I	
727/I	Petrova Gora	CC	B	+
822/I	Petrinja	CC	B	+
888	Petrova Gora	CC	B	+
985	Petrinja	SN	B	+
996	Istra, Buje	CC	B	+
694	Glina, hybrid	CC	I	+
793	Glina	CC	I	+
822/II	Petrinja	CC	I	+
1004	Glina	CC	I	+

*Culture characteristics:
1. Normal with abundant pycnidia (N)
2. White; poor or reduced sporulation (B)
3. Intermediary characteristics (I)

The dsRNA in our strains showed one band of identical mobility to that of dsRNA in Swiss and Italian strains, which means that Swiss, Italian and Croatian strains may contain similar dsRNA (Rigling, personal communication).

CONVERSION OF VIRULENT ISOLATES

The experiments of hypovirulence transmission were made by pairing compatible virulent and hypovirulent isolates on PDAmb. After a few days, the vegetatively compatible pairs reacted by the merging of mycelia with subsequent changes of virulent isolates to less pigmented or white mycelium. The converted mycelium was transferred to fresh PDAmb and used for pathogenicity tests on excised chestnut stems. Results of these tests, 5 wk after inoculation, showed small developing canker areas of converted virulent isolates (data not shown). However, the canker area values of converted isolates, except isolate No. 4-727/11, were larger than those of other hypovirulent isolates.

CONTROL OF CHESTNUT BARK CANKER

Our first experiments to control chestnut bark cankers were carried out in 1983-1984 with French hypovirulent strain No. 2 022, and with our white strain 737/11 isolated from a very developed, callused canker in Petrova Gora. A total of 50 trees were inoculated; 40 with the French hypovirulent strain and 10 with our 727/11 isolate. Inoculations were carried out by removing a plug of bark and placing mycelium into the hole with a spatula. Every inoculation hole was covered by microporous tape. Development and change of inoculated cankers were recorded every year until 1988. Stagnation of inoculated-canker development was observed on 22 trees during the first year. The virulent fungus overcame the hypovirulent inoculation, as determined by the sunken and changed color of the bark. On some of these progressive cankers, fruiting bodies appeared. Since 1988,11 trees have died. Cankers on the remaining trees gradually stopped expanding and the callus tissue increased in size. The canker margins were superficially necrotic, typical for hypovirulent strains. Even though the treatments with hypovirulent strains were made around cankers caused by strains of unknown vegetative compatibility, 78% of the cankers were controlled.

A second experiment to control chestnut blight was carried out at the beginning of July 1988, with a mixture of 12 hypovirulent isolates. The mixture was composed of isolates that individually, in pathogenicity tests, showed reduced canker areas; dsRNA was confirmed for six of the isolates. Inoculations were made around cankers on 42 chestnut stems grafted with Marrone cultivars. The diameters of stems were approximately 4-8 cm and cankers varied in size. The number of inoculated plugs was 3-6,

depending on canker size. Inoculations were protected with microporus tape as on excised stems. Measurements 3 mo later showed that canker development was arrested in 36 of 42 treated trees, and six trees had died. By the autumn of 1990, only 26 trees remained alive; the remaining trees had died due to C. *parasitica.* The dead trees were smaller in diameter than survivors. Cankers had stopped expanding on surviving trees before fructifications and water sprouts had formed.

CONCLUSION

Chestnut blight was spread throughout the natural distribution of chestnut in Croatia. In many chestnut forests, hypovirulent forms of the pathogen have been found. Hypovirulent strains appeared spontaneously and have the ability to spread naturally. Hypovirulent strains are converting virulent strains, slowing canker growth, and allowing trees to compartmentalize and produce callus tissue.

Taking into account the slow rate of natural dissemination of hypovirulent strains, and in an attempt to increase their occurrence in chestnut stands, it is recommended that: (1) attempts be made to preserve the trees that exhibit symptoms of hypovirulence; (2) mixtures of hypovirulent strains be introduced into forests where hypovirulence is not yet found and new hypovirulent strains be isolated; and, (3) hypovirulent mixtures be created that will be more efficient in transmission and conversion of the virulent strains threatening the chestnut trees of Yugoslavia.

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