Some Aspects of Natural Spread of Hypovirulence in Italy*

Tullio Turchetti

Centro di Studio per la Patologia delle Specie Legnose Montane del C.N.R., Istituto di Patologia e Zoologia Forestale e Agraria, dell'Universita di Firenze, Piazzale della Cascine, 50144 Firenze, ITALY

ABSTRACT. The spread of hypovirulence and the associated presence of healing cankers is naturally increasing in many chestnut stands in different regions of Italy. The data collected during three years of observations of new infections in some chestnut plots, indicate the slow natural spread of hypovirulent isolates. Pycnidia production was observed on the surface of these cankers during their initial development. Encouraging results have been obtained in artificial inoculations with a combination of four hypovirulent isolates that sporulate well. These isolates are able to convert virulent isolates of *Cryphonectria parasitica* belonging to 10 vegetative compatibility groups, and have been used to cure virulent cankers. The biological control of blight in the chestnut orchards cultivated for fruit production is emphasized in this study.

Two events have renewed interest in chestnut trees in Italy: their regrowth after the severe damage caused by *Cryphonectria parasitica* (Murr.) Barr and the price of chestnuts. The natural establishment of hypovirulent isolates, able to cause healing cankers, has resulted in this improvement in the control of chestnut blight (3, 4, 5, 8, 12, 15). These factors have encouraged the cultivation of chestnut trees in many orchards.

The objectives of the present study were to verify the presence of hypovirulence of *C. parasitica* in chestnut stands located in different regions of Italy and collect preliminary data on the natural spread of virulent and hypovirulent isolates of *C. parasitica.*

MATERIALS AND METHODS

Field observations were carried out in different chestnut stands located in the following regions of Italy: Piedmont, Trentino-Alto Adige, Tuscany, Marche, Campania and Sardinia. The purpose of the investigations was to verify the presence of abnormal cankers produced by hypovirulent isolates of *C. parasitica.* Samples of normal and abnormal cankers were collected in each stand.

Four chestnut stands were located near Florence: Montecuccoli, Montesenario, Pomino and San Donato in Collina. One experimental plot was selected in each stand that appeared to be established exclusively by chestnut sprouts. The average ages of the chestnut sprouts in each plot were 15, 30, 7 and 30 yr old, respectively. An incremental borer was used to determine the ages of the chestnut sprouts.

At each study site a clump of 36 chestnut stumps was selected as the plot. In each plot, all the chestnut sprouts of each stump were numbered with red paint and each stump was labelled with a metal tag. Between 1989 and 1991 all the marked chestnut sprouts were rated yearly for each of the following: dbh (1.4 m above ground) of all the stems over 3 cm dbh, live stems with or without chestnut blight, dead stems, presence of new infections and cankers on each stem. These ratings were done just before budbreak each spring.

Samples of normal and abnormal cankers for isolation were collected in each experimental plot. *C. parasitica* was isolated by placing small pieces of bark tissue bark on Potato Dextrose Agar (Difco, Detroit, Mich.) amended with methionine, 100 mg/1 and biotin, 1 mg/1 (PDAmb). All the hypovirulent (H) and virulent (V) isolates were subcultured and tested on PDAmb. Tests for vegetative compatibility were made as described in Anagnostakis et al. (1). All subcultured isolates were transferred to plates of PDAmb and paired with each other and with vegetative compatibility testers in the CSPSLM collection. Test pairs were incubated at 25-27 C in the dark for 15 days. If results were unclear, pairings were repeated.

Hypovirulence conversion tests were conducted according to the methods of Turchetti and Maresi (17). Four dsRNA-containing *C. parasitica* isolates, previously studied by Turchetti and Maresi (15) were used as known H isolates in the conversion tests. Inocula of the isolates were transferred to plates of PDAmb and paired. Pairings were incubated at 25-27 C in the dark for 15 days. Small disks (maximum 3-mm-diameter) of V mycelium were transferred to fresh PDAmb plates and examined for the white appearance of colonies, characteristic of H strains.

Ten chestnut sprouts were inoculated in a chestnut stand at Montesenario with the set of 4 H strains to test their ability to limit the growth of 10 V isolates (one H isolate for each v-c group). The stems were treated with the method described by Turchetti and Maresi (17). The treatment was replicated three times for each sprout. The controls were: wounds without added inoculum, and wounds inoculated with a V strain. Inoculations were performed in the spring of 1990 and canker development was examined after 1 yr.

RESULTS

The presence of abnormal (H) cankers was detected in all the chestnut stands in each of the six Italian regions

*This research was supported by the Italian Ministry of Agriculture and Forestry under the project "Biological and Integrated Control of Forest, Trees and Crops," subproject "Forest Trees."

(Table 1). In the provinces of Piedmont, Tuscany, Marche and Campania (Avellino) their frequency was high (between 58% and 87%). On the contrary, the percentage of normal (V) cankers was greater than 80% in some chestnut stands located in Oristano (Sardinia) and Bolzano (Alto Adige). Abundant pycnidia formation was observed on the cankered bark in these stands. Perithecia also were present. In the stands with high incidences of abnormal cankers, very few perithecia were observed. Normal cankers were predominant (between 54% and 67%), but a large number of natural abnormal or superficial infections was present in the sites near Salerno (Campania), Nuoro (Sardinia) and Trento (Trentino Alto Adige).

Based on species present, some sites located near Salerno (Campania) appeared to be former oak-chestnut woods. Hornbeam *(Ostiya carpinifolia* Scop.) also were present. In these plots, cankers caused by *C. parasitica* were observed on *Quercus ilex* L. and *Q. pubescens* (Thuill.) Lamk. Small oak trees were found that had been killed by C. *parasitica.* Infections of the pathogen also were discovered on *0. carpinifolia.* V isolates were exclusively obtained from the cankers on these hosts (18).

New infections appeared to be "normal" because of cracks that penetrated to the cambium, epicormic sprouts below the canker and the abundant stromata. The growing cankers girdled the trees killing the branch or stem distal to the cankers. Three different types of abnormal cankers were found. The appearances of the first and second types were similar to those of "abnormal cankers" on *Castanea dentata* (Marsh.) Borkh. in the USA (6, 10). The second type of canker had numerous, superficial, regular cracks in the bark and had active pycnidia production. The third type of abnormal canker was completely swollen and healed. A more or less pronounced swelling characterizes all these cankers. Cankers intermediate between normal and abnormal types also were found.

Tunnels produced by some corticolous insects Esch. and others) were frequently observed on the bark of the young stems. At the end of these tunnels, the cuticle appeared swollen and a large surface of periderm was exposed to the infections of the parasite. In some instances, the tunnels were colonized by *C. parasitica* mycelium.

Dieback of stumps was observed in some stands located in Piedmont, Marche, Tuscany and Sardinia. Many dead stems had intact bark with no sign of chestnut blight. Symptoms of the "ink disease" *(Phytophthora cinnamomi* Rands) were observed at the bases of the trunks and sprouts.

During the 3 yr of the study, the percentage of sprouts with cankered stems increased yearly in all plots (Table 2). In the old sites (more than 30 yr old) located at San Donato in Collina and Montesenario, the percentage of the blighted sprouts was high *(75%* and 81%). In the other two study sites (Montecuccoli and Pomino), 15 and 7 yr old, respectively, the percentage of cankered stems was not as high (about 45% and 50%).

The percentage of sprouts with canker-killed stems was high at Montesenario (more than 25%) and this value remained about the same during the course of the study. Sunken cankers with abundant stromata were present on the blighted living stems. Such lethal cankers resulted in the death of many stems during the three years of the study. There were few apparent superficial or abnormal cankers in this plot. Survival of sprouts was related to stem growth (10, 14, 16). Although blight incidence was high among the largest chestnut stems (2 or more cankers per stem), they were able to survive. On the contrary, smaller and subcanopy stems were frequently blighted and killed by the pathogen.

In the other three sites surveyed, the percentage of blight-killed stems was low (5%). In these chestnut plots, a high incidence of abnormal cankers was observed and their number increased each year (Table 3). Chestnut sprout survival was greater than 75% in the plot at San Sonato in Collina.

The incidence of new infections appears to be low even though the number of cankers was high in all the surveyed plots (Table 3). During 1990, a large number of new attacks occurred at Pomino, the sites with the youngest trees.

Isolates of *C. parasitica* were obtained from the samples collected in the chestnut stands. A high incidence of H isolates was detected in the stands where a prevalence of abnormal and superficial cankers was observed (Table 1). A large number of V isolates was obtained from the more chestnut blight-damaged areas located in Alto Adige and Sardinia. Intermediate cultures were isolated from cankered samples collected in all the sites recognized.

Cryphonectria parasitica V isolates (total 118) tested for v-c groups could be assigned to one of the ten v-c groups found in Italy. Vegetative compatibilty group 1 was common and found in all of the regions. Vegetative compatibility groups 2, 4 and 5 were the types most frequently recovered (Table 1). Two v-c groups (9 and 10) were found exclusively in Sardinia.

There was transmission of hypovirulence as a result of conversion between the four H isolates tested and all 10 v-c groups (Table 1).

When H isolates were applied around cankers of sprouts inoculated with V isolates, the H infections were effective in reducing canker size (Figure lb). Cankers from V strain inoculations that received no treatment with H isolates, grew until they girdled and killed their sprouts.

DISCUSSION

The results of this investigation indicate that hypovirulence is spreading in the six Italian regions studied. Trees and sprouts were regrowing in all the stands and the old dead branches were an indication of the previous severity of this disease. Small blight-killed shoots with leaves still attached were seen in the crown of many chestnut trees. These observations indicate that the V strains are primar-

Table I. Chestnut blight in six regions of Italy: Incidence of abnormal cankers, isolate morphology, vegetative compatibility groups of *Cryphonectria parasitica* and virulent isolates converted.

Regions	Provinces	Percent Cankers		Isolates*				Number of Isolates in Each Vegetative Compatibility Group										V Isolates
		Normal	Abnormal	V	I	W	Total	1	2	3	4	5	6	7	8	9	10	Converted**
Piedmont	Turin	29	71	3	14	20	37	1	1				1					+
Trentino	Cuneo	42	58	3	7	3	13	2				1						+
Alto Adige	Trento	67	33	27	8	5	40	9	4	3	4	5		1	1			+
	Bolzano	92	8	25	2	3	30	3	11	1	7	1	1					+
Tuscany	Arezzo	13	87	4	4	17	25	1	1		1		1					+
	Florence	22	78	6	3	10	19	2			1	1	1		1			+
	Grosseto	17	83	2	4	6	12	1			1							+
	Lucca	28	72	3	6	9	18	1	1		1							+
Marche	Pesaro	48	52	6	3	8	17	2	1		1	1	1					+
Campania	Avellino	22	78	3	7	16	26	1			1	1						+
	Salerno	55	45	18	15	7	40	5	7	4		1		1				+
Sardinia	Nuoro	54	46	13	9	7	29	1	2	1	2	5				1	1	+
	Oristano	81	19	5	1		6			1	1			2		1		+

*V = Virulent isolates, I = Intermediate isolates, W = White isolates
**vc = vegetative compatibility
†Number of isolates in each vc group

Table 2. Percentage of blighted and killed sprouts in four chestnut plots.

Plot	Age of the sprouts	1989*			1990			1991		
		A**	B	T	A	B	T	A	B	T
Montecuccoli	15 years	36†	1	37	40	2	42	42	3	45
Montesenario	30 years	45	25	70	47	25	72	49	26	75
Pomino	7 years	42	2	44	40	5	45	45	5	50
San Donato in Collina	30 years	71	2	33	77	3	80	76	5	81

*The same plots were surveyed for three successive years
**A = blighted sprouts; B = killed sprouts; T = total
†Percentage

Table 3. Cankers and new infections recognized in the experimental plots.

Plots	1989*			1990			1991		
	N.I.**	A	N	N.I.	A	N	N.I.	A	N
Montecuccoli	13†	126	4	17	134	9	25	146	14
Montesenario	9	57	78	13	61	83	13	69	87
Pomino	23	104	13	43	125	15	37	164	19
San Donato in Collina	9	242	22	20	259	24	16	276	35

ily limited to colonizing young or weak branches. Many surviving branches in the observed trees were blighted and many living sprouts had cankered stems. In general, where the percentage of surviving cankered branches or sprouts was high, the number of abnormal cankers also was high. Superficial cankers were most frequent in the managed chestnut orchards for fruit production and in plantations plots. Like Griffin et al. (10, 11), we also observed that clearcuts can increase the frequency of superficial cankers in coppices.

Many isolates with abnormal morphology (62%) were recovered from the superficial cankers collected, which indicates that dsRNA is common in *C. parasitica* strains in many Italian chestnut stands. Natural spread of dsRNA within and among isolates of *C. parasitica* is occurring, as referred to by Garrod et al. (7) and Anagnostakis (2). The dissemination of hypovirulence and the transmission of dsRNA to new generations of the chestnut blight fungus appears to be caused by the production of conidia-containing dsRNA. Hypovirulent inoculum is produced by cirrhi and pycnidia that are present on the surface of abnormal cankers during their initial development. The relative numbers of these cankers are important for efficient spread of H inoculum. Cankers on younger stems are more active in producing *C. parasitica* inoculum than are the numerous swollen and healed infections present in the older sprouts. The level of new infections was low in the experimental plots where there is a prevalence of V

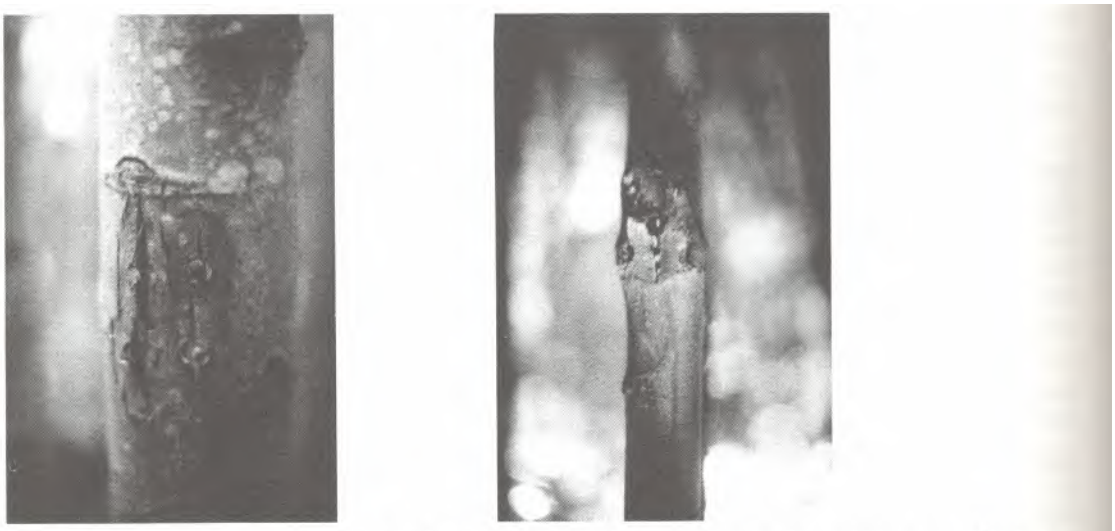

Figure 1. Healing canker produced by: (a) the combined inoculation of the four A strains; pycnidia are produced, (b) inoculation around V infection.

isolates. Reduced perithecia production is another factor favorable to the effectiveness of hypovirulence and the regrowth of chestnut trees in Italy. Different biotic or abiotic vectors may be involved in the natural spread of hypovirulence: wind, rain, birds, mammals, insects, mites and slugs. Further research is needed to determine the role of mites in the dissemination of *C. parasitica* (9, 13). Observations also are being carried out on the role of corticolous insects in the dispersal of the fungus (Tiberi and Turchetti, unpublished).

Because of the low incidence of new infections, it requires many years (10-15) for the establishment and prevalence of the hypovirulent isolates in abandoned chestnut stands. In these stands, the ratio between new infections and cankers was between 1:6 and 1:10.

In orchards, the presence of many abnormal cankers is important in order to increase the establishment and dissemination of hypovirulent isolates. This goal can be achieved by pruning blight-killed branches or sprouts and artificially introducing H isolates. Treatments with four H isolates in combination on V infected branches and sprouts were able to produce healing cankers (Figure lb) (17). The transmission of dsRNA to other strains of *C. parasitica* occurs through hyphal anastomosis. The effectiveness of treatment of cankers with H isolates is enhanced by the relatively few vegetative compatibility groups of the fungus found in the Italian regions studied. Pycnidia appear on the bark of these healing cankers and sustain the prevalence of the hypovirulent isolates in the treated chestnut stands. Biological control of chestnut blight is enhanced by inoculating with combinations of H strains. Programs of artificial inoculations are being carried out in Alto Adige and Sardinia where a prevalence of V isolates was verified. Further studies are needed in mixed forests where chestnuts are associated with different species of tree.

ACKNOWLEDGMENT

I thank Dr. G. Maresi for his collaboration.

LITERATURE CITED

1. Anagnostakis, S.L., Hau, B. and Kranz, J. 1986. Diversity of vegetative compatibility groups of *Cyphonectria parasitica* in Connecticut and Europe. Plant Disease 70:536-638.
2. Anagnostakis, S.L. 1990. Improved chestnut tree condition maintained in two Connecticut plots after treatments with hypovirulent strains of the chestnut blight fungus. For. Sci. 36:113-124.
3. Bonifacio, A. and Turchetti, T. 1973. Differenze morfologiche e fisiolopgiche in isolati di *Endothia parasitica.* Annali Accademia Italiana Sc. Forest. 22:111-131.
4. Elliston, J.E. 1981. Hypovirulence and chestnut blight research: fighting disease with disease. J. For. 657-660.
5. Falcini, L. and Bisiach, M. 1978. Patogenicità di *Endothia parasitica* e possibility di lotta biologica contro it cancro della corteccia del castagno. II Montanaro d'Italia 3:65-70.
6. Fulbright, D.W., Weidlich, W.H., Haufler, KZ., Thomas, C.S. and Paul, C.P. 1983. Chestnut blight and recovering American chestnut trees in Michigan. Can. J. Bot. 61:2164-3171.
7. Garrod, S.W., Fulbright, D.W. and Ravenscroft, A.V. 1985. Dissemination of virulent and hypovirulent forms of a marked strain of *Endothia parasitica* in Michigan. Phytopathology 75:533-537.
8. Grente, MJ. and Sauret, S. 1968. L'hypovirulence exclusive, phenomene original en pathologie vègêtale. C. R. hebd. Seanc. Acad. Sci. Paris 268:2347-2350.
9. Griffin, GJ., Wendt, R.A. and Elkins, J.R. 1984. Association of hypovirulent *Endothia parasitica* with American chestnut in forest clearcuts and with mites. (abstr.) Phytopathology 74:804.
10. Griffin, GJ. 1989. Incidence of chestnut blight and survival of American chestnut in forest clearcuts and neighboring understory sites. Plant Disease 73:123-127.
11. Griffin, GJ., Smith, H.C., Dietz, A. and Elkins, J.R. 1991. Importance of hardwood competition to American chestnut survival, growth and blight development in forest clearcuts. Can. J. Bot. 69:1804-1809.
12. Jaynes, R.A. and Elliston, J.E. 1980. Pathogenicity and canker control by mixtures of hypovirulent strains of *Endothia parasitica* in American chestnut. Phytopathology 70:453-456.
13. Nannelli, R. and Turchetti, T. 1989. Osservazioni preliminan sull'asssociazione di alcune specie di acari corticicoli con *Cryphonectria parasitica* (Mum) Barr. Redia, LXXII 2:581-593.
14. Turchetti, T. and Marinelli, A. 1980. Rilievi preliminari sul cancro corticale del castagno in alcuni cedui della provincia di Pistoia. L'Italia forestale e Montana 35:80-90.
15. Turchetti, T. and Maresi, G. 1988. Mixed inoculum for the biological control of chestnut blight. Bulletin OEPP/EPPO 18:67-22.
16. Turchetti, T. and Maresi, G.1990. Indagini sulla diffusioner naturale degli isolati ipovirulenti di *Cryphonectria parasitica* in alcuni cedui di caastagno. Atti Giornate Fitopatologiche 2:89-98.