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Chestnut blight: an epidemic checked by biological control

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Abstract. Chestnut blight is one of the most devastating plant diseases. Within 50 years of its discovery in North America in 1904, mature American chestnut trees (*Castanea dentata* Borkh.) could only rarely be found within their natural range and the tree now survives only as sprouts growing from stumps of trees attacked by the blight. The disease was then introduced into Europe where it caused considerable damage in the local chestnut (*Castanea sativa* Mill.) plantations. However the development of biocontrol techniques, based on natural recovery of diseased plants, has prevented the extinction of the European chestnut. The history of the epidemics, the use of hypovirulent strains of the pathogen for biological control and the work carried out by the author and his co-workers are reviewed and discussed.

Keywords. Chestnut blight, Biocontrol, *Cryphonectria parasitica*, dsRNA, Hypovirulence, Vegetative compatibility.

Introduction. When plant pathogenic fungi develop for a long time together with their specific hosts, the association often results in a sort of equilibrium, where growth and reproduction of both partners is assured and consequently rarely catastrophic epidemics result. The picture changes completely when new hosts or, even worse, new pathogens are introduced into a particular environment. In most cases the results are disastrous, as shown for example by the arrival in Europe of *Phytophthora infestans* in potato crops and by *Plasmopara viticola* or *Oidium tuckeri* in vine cultures. By the constant and appropriate use of fungicides it is possi-

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ble to keep these diseases under control, although the economic and ecological impact cannot be underestimated.

A further example is the introduction, first into the USA and then into Europe, of the chestnut blight fungus. At the beginning of the last century only a fe the USA, probably with Asian chestnut plants (Anagnostakis 1987), it had almost completely wiped out the American chestnuts (*Castanea dentata* Borkh.) of the Eastern states. The disappearance was almost total: "once one of the most valuable of American hardwoods, this species has been virtually destroyed […] few uninfected trees exist, though stump sprouts persist" (Brockman 1968). The disease caused significant problems in Europe when it arrived a few decades later and a similar fate loomed over the European chestnut (*Castanea sativa* Mill.). However it was observed, around the middle of the 1950s, that some diseased plants recovered naturally thus saving the European chestnut from extinction (Heininger & Rigling 1994). This led to the development of biocontrol techniques capable of counteracting the disease, at least in Europe.

The following report deals both with the history of the dynamics of chestnut blight and relative work carried out by the author and his colleagues at the universities of Milan and of Udine.

Historical background

The host. In America chestnut trees [*Castanea dentata* (Marsh.) Borkh.] were a major component of the hardwood forests of the eastern States (Gravatt 1949). The European host is a related species, *Castanea sativa* Mill. introduced from Asia Minor by the Romans (Huntley & Birks 1983). In Europe chestnuts are grown as coppices, with a rotation period of 15-30 years, and in woods and orchards. Quite old trees, grafted with local varieties, are common (Heiniger & Rigling 1994). In both continents the trees were of great economic importance. Nuts were an important source of food for humans, wildlife and domestic livestock. Trees provided timber, firewood and tannin. The chestnut wood seasons well and is resistant to decay because of the tannins present both in the bark and in the wood (Nienstaedt 1953). It was used for construction, furniture, fencing, boxes, barrel staves, railroad ties, telegraph poles, mine timbers and even musical instruments (Rice et al. 1980). The stature of the trees made them useful, at least in the USA, in urban planting (Emerson & Weed 1918), and it was on the chestnuts trees lining the avenues of the New York Zoological Gardens that the blight was first detected in 1904 (Merkel 1905). The tannins were extracted both from the bark and the wood and formed the basis of a large leather tanning industry (Fowler 1944, Saucier 1973).

The social consequences of the disappearance of the chestnut cannot be evaluated simply in economic terms, chestnut trees are still important in marginal areas, also ecologically. Following the abandonment of landby mountain farmers, unprof-

itable areas are subject to soil erosion and degradation. Chestnut trees are a safeguard in these habitats.

The pathogen

The causal agent of the chestnut blight (the fungus however is also found in trees of the genus *Quercus*) and was first described by Murrill (1906) under the name *Diaporthe parasitica*. A few years later, Anderson and Anderson (1912) transferred the species to the genus *Endothia* and finally, following her re-evaluation of the *Diaporthales*, Barr (1979) renamed the genus *Cryphonectria* (Anagnostakis 1987). The present nomenclatural status of the fungus is therefore *Cryphonectria parasitica* (Murr.) Barr.

However, apart from taxonomists' quibbles over nomenclature, other issues, such as the biology of the fungus and its spread, deserve more attention.

Biology of the fungus. Cryphonectria parasitica is an ascomycete that produces perithecia deeply buried in stromata and with long necks terminating at the surface with an ostiole. The asci are club-shaped (7-9 x 30-60 µm) and no paraphyses are present. The ascospores (3.5-5 x 7-11 µm) are two-celled, hyaline and with round ends. At least in Italy perithecia can be found more frequently in the autumn (Pratella 1978). The asexual stage of the fungus is represented by conidia produced in orange-red pycnidia. Inside the pycnidium, conidia are formed at the pointed apex of simple or branched (1.5 by 10 µm, or longer) conidiophores. Conidia, hyaline, unicellular, cylindrical (1.3 x 3.6 µm), sometimes slightly curved, and with rounded ends, become detached and crowd together inside the pycnidial cavity from which they issue in spiral-like cirri, held together by a mucilaginous material. Pycnidia are formed in various periods of the year and also on old dead branches (Pratella 1978).

Origin of the pathogen. The available data on the origin of the fungus is summarised by Anagnostakis (1987) as follows. Because chestnut trees from China and Japan show resistance to the disease, early researchers believed that *C. parasitica* originated in those locations (Metcalf 1908, Clapper 1952). Requests for information to scientists in those countries proved fruitless, however the same fungus as that causing the blight disease was isolated from samples collected in 1913 by explorers (David Fairchild and Frank Meyer) in China. Identification was confirmed by inoculating American chestnut trees near Washington, D.C. A few years later Meyer found the disease in Japan and sent samples to the United States (Fairchild 1913, Shear & Stevens 1913, 1916).

Dissemination. At first it was been suggested (Murrill 1906) that the conidia of the fungus were dispersed by wind of conidia. Being linked into tendrils, they obviously cannot be carried far by wind. Conidia however do remain viable in the soil for a long time and might be dispersed by windborne dust (Heald & Gardner 1914).

On the other hand ascospores are actively expelled from their asci. Heald et al. (1915) showed that drying of bark after rain allowed ascospore discharge to continue for up to 14 hours. Following dry periods, as little as 4.5 mm of rain was sufficient to cause an abundant expulsion of ascospores.

Conidia have been recovered in large numbers from the surfaces of animals. Several studies on insect carriers, dating back to the arrival of the pathogen in the USA (Craighead 1912, Studhalter 1914, Studhalter & Ruggles 1915) as well as more recent ones (Wendt et al. 1983, Russin et al. 1984) point out that conidia are more frequently recovered from the surface of insects than ascospores and that the most important insect carriers are probably *Strophiona nitens* Forster, *Ectoedema phleophaga* Bsk. and *Leptostylus maculata* Say. As they move over canker stromata birds and small mammals also pick up *C. parasitica*., as demonstrated from washings of the animals (Heald & Studhalter 1914, Scharf & De Palma 1981).

Man contributes with the transport of plants and wood. Infected and not debarked logs of *Castanea* and *Quercus* harbour fungal mycelium, pycnidia, and perithecia. Nutshells too can also be infected (Jaynes & De Palma 1984).

The disease

Symptomatology. Cryphonectria parasitica is a wound pathogen. It infects branches and stems, grows in the bark and into the cambium and causes bark cankers. Girdling of branches induces wilting and dieback of the distal parts. Roots are not infected and stumps sprout repeatedly. The most characteristic symptomatology is present on woody parts of the plant and vary according to the stage of differentiation of the latter. Shoots and very young branches show irregular brick-red spots with the tissues at the border slightly raised. A creamy mycelial mass can be observed under the latter. On more mature, but incompletely lignified branches, similar spots, though more developed longitudinally and slightly depressed, are present. As the infection progresses it girdles the branches which frequently show hypertrophic growth with longitudinal cracks. The typical cankers develop on lignified branches. The cortical tissues show deep cracks and are easily detachable. Underneath a creamy fan-like or radiating stromatic felt of mycelium is present. Infected plants are easily detectable also from a distance by the dry leaves remaining attached to the twigs and the abundant shoots produced below the cankers (Pratella 1987).

Spreading of chestnut blight. As mentioned before the disease was first seen on American chestnut trees in the New York Zoological Gardens in 1904 (Merkel 1905). Very shortly afterwards *C. parasitica* moved out of New York State. It progressed rapidly through the entire native range of *Castanea dentata,* from Maine to Alabama, and west to the southern border of Michigan. Spreading at the rate of about 40 kilometres per year, within 50 years about 3.6 million hectares of American chestnut trees

had been killed or were dying (Anonymous 1954) and the epidemic could not be stopped. Pruning and spray programs using Bordeaux mixture failed to control or contain the disease (Murrill 1906). State foresters could do little but suggest possible uses for the dead timber and the length of time it could be expected to remain sound and marketable (Anagnostakis 1987).

The official entry of *C. parasitica* into Europe can be dated back to 1938 (Biraghi 1946). The initial infection foci were detected in the Liguria Apennines around Genoa, one of the main Italian ports for merchant ships, and also in the province of Udine and in a small area around Avellino in the south. However it spread very rapidly and most chestnut trees became affected by the end of the forties in northern Italy (Baldacci & Picco 1947). By 1950, Biraghi found blight widely distributed in the northern and southern chestnut-growing regions. He recommended cutting down the dead trees at ground level to reduce the amount of inoculum (Biraghi 1953). According to some authors (Del Guerra 1948), the blight had been introduced much earlier but had gone unnoticed. During the 1920s *Castanea crenata*, the Japanese chestnut, resistant to ink disease, was planted in Italy and other European countries. *Cryphonectria parasitica* is a weak pathogen of Asian chestnuts and was probably imported on that occasion.

From Italy the disease spread into adjacent countries (Table 1). Chestnut blight also went unnoticed for a long time in some areas because of decline due to ink disease and frost damage (Heiniger & Rigling 1994).

Table 1. The chestnut blight epidemic in Europe (after Heiniger and Rigling, 1994) (A: First observation of *Cryphonectria parasitica*; B: First observation of healing cankers; C: Isolation of hypovirulent strains; D: Number of vegetative compatibility groups (VCG); ND: not detected).

Country	A	B	C	D
Italy	1938	1951	1964	>17
Spain	1947	1992	1992	> 5
Switzerland	1948	1975	1975	> 6
Croatia	1950	1978	1981	8
France	1956	1964	1964	>20
Greece	1964	1975	1984	2
Hungary	1965	ND		
Turkey	1967	ND		
Albania	1967	1983	1984	
Austria	1970	1993	1993	> 7
Slovakia	1976	ND		2
Portugal	1989	ND		
Germany	1992	1993	1993	2

According to some authors *Castanea sativa* is slightly more resistant to blight than *C. dentata*. This lengthens the time from infection to death, and may have an effect on inoculum potential (Anagnostakis 1987). In addition the spread of the disease in the old world was not particularly fast, because of non continuous chestnut stands in Europe.

Development of hypovirulence

Initial observations. In view of what had happened in the USA and considering the similarity with the epidemics in Italy, it was easy to assume a similar fate awaited the European chestnut trees. However at the end of the second World War and just 15 years after the entry of the pathogen into Italy, Biraghi (1953) discovered a 'surprisingly healthy' chestnut coppice, despite heavy (85%) infection of the shoots. Upon close examination, some cankers appeared atypical, lesions stopped spreading round the trunks and trees started to recover spontaneously. The atypical cankers were restricted to the outer layer of bark, they eventually healed and were comparable to those observed earlier on (Biraghi 1949) in the bark of *Castanea crenata*. In 1954, he reported that trees were recovering 'in many parts of Italy where the pathogen has been present for a certain number of years' (Biraghi 1954). Biraghi (1953) attributed the phenomenon to increased resistance of the trees, induced by repeated cutback of the sprouts.

Isolation of hypovirulent strains. On a visit to Italy in 1964 with a group of FAO foresters, Grente took some bark samples from recovering trees growing in the Como and Varese provinces (northern Italy). From these he isolated forms of the blight fungus that looked different and had reduced virulence (Grente 1965). Similar strains were subsequently found in France and other European countries (Table 1). The hypovirulent strains showed abnormal features. They grew slowly and erratically on potato dextrose agar (PDA), colonies were whitish, rather than orange, in colour and produced significantly fewer conidia.

Nature of hypovirulence. When chestnut trees were wound-inoculated, the atypical strains showed reduced virulence. Moreover, this low virulence (*hypovirulence*) was transmitted to virulent strains during hyphal anastomosis when cultures were grown on agar media (*transmissible hypovirulence*), converting them to hypovirulence. When the expanding margins of cankers in the field were inoculated with hypovirulent strains, the former stopped growing and only hypovirulent strains could be isolated, and finally when atypical strains were co-inoculated with virulent isolates most of the resulting cankers healed.

Hypovirulence, as well as cultural characteristics (pigmentation and reduced sporulation), were shown to be cytoplasmically controlled (Grente and Sauret, 1969; Van Alfen et al. 1975) and associated with high molecular weight double-stranded (ds) RNAs (Day et al. 1977). Hypovirulent strains could convert virulent

strains to hypovirulence by the transfer of dsRNA via hyphal anastomosis (Anagnostakis 1981, Anagnostakis & Day 1979, Rigling et al. 1989). These findings formed the basis for the development of biological control of the disease.

Hypovirulence-associated symptoms. As indicated previously hypovirulent strains exhibit hypovirulence-associated symptoms, such as altered colony morphology, suppressed conidiation and reduced oxalate, laccase and pigment production (Anagnostakis 1982, 1984; Havir & Anagnostakis 1983, Rigling et al. 1989). Some of the differences are influenced by environmental factors. Pigmentation, conidiation, and oxalate production are suppressed when virulent *C. parasitica* strains are grown in the dark (Pullhalla & Anagnostakis 1971) and suppression of the syndrome can be partially achieved by exposure to high light intensity (Hillman et al. 1992). At the same time conidiation and pigmentation are not significantly suppressed in some American strains (Nuss & Koltin 1990). Following the initial characterisation, hypovirulent strains exhibit a wide range of associated symptoms. At present it seems that associated characters are rather dependent on the specific action of dsRNA-encoded gene products.

dsRNA as the causal agent of hypovirulence. The hypovirulent phenotype is associated with cytoplasmic genetic elements that are transmitted during hyphal anastomosis (Van Alfen et al. 1975) The genetic information responsible for transmissible hypovirulence resides on cytoplasmically replicating dsRNAs, presumably of viral origin (Day et al. 1977, Fulbright 1984, Elliston 1985). Hypovirulent strains contain dsRNA, whereas virulent have no dsRNA. Electron micrographs show that the dsRNA is not encapsidated but associated with membrane-like vesicles in the cytoplasm (Newhouse et al. 1983) and that, in contrast to many virus-like particles (VLPs), it occurs mainly in the apical zone of *C. parasitica* hyphae.

Mycoviruses and related double-stranded (ds) RNA genetic elements are associated with fungi at a high frequency (Nuss & Koltin 1990). Fungal viruses were first detected in cultivated mushrooms (Hollings 1962) and a few years later in *Penicillium funiculosum* (Banks et al. 1968). In addition to encapsidated viral dsRNA, unencapsidated dsRNA elements have also been found in all major groupings of the Fungi kingdom (Nuss & Koltin 1990). In fungi, viral dsRNA genomes are mostly segmented and each dsRNA fragment is separately encapsidated. The unencapsidated dsRNAs are similarly represented by multiple segments, but associated with cell membranes (Van Alfen 1987).

Hypovirulent strain dsRNAs (Nuss 1992) are extremely variable, with lengths falling into three broad size ranges, namely S (small), M (medium) and L (large). They all show the same terminal regions: a polyA tail at the 3' end and a 28-nucleotide conserved sequence at the 5'

end. They differ in the degree of internal deletion of the molecule. The large form appears to be the full-length molecule and the smaller forms seem to be defective derivatives. The high degree of variability of RNA genomes is not unusual because there is no effective proof-reading system for RNA genomes, to ensure their faithful replication (Deacon 1997).

The L-dsRNA (about 12.7 kbp) contains two continuous coding domains [ORFA of 622 codons (nucleotide triplets) and ORFB of 3165 codons] and multiple defective interfering segments (Shapira et al. 1991a, 1991b). The defective dsRNAs are internally deleted forms of the large dsRNA, generated by subculturing, transfer of the L-dsRNA via hyphal anastomosis, and after passage of the fungus through host tissue (Heiniger & Rigling 1994). The contribution of the defective dsRNAs to the expression of the hypovirulent phenotype is not yet clear.

Genetic organisation, expression and replication are strongly suggestive of the potential viral origin of the dsRNA (Shapira et al. 1991a, Fahima et al. 1993). The introduction of L-dsRNA into *C. parasitica* by DNA-transformation has established that dsRNA is the causal agent of hypovirulence (Choi & Nuss 1992). With the development of a transformation system, Nuss and his colleagues (Hillman et al. 1989, Nuss & Koltin 1990) were able to produce a cDNA from the full-length dsRNA. The cDNA could also be used for molecular analysis of the dsRNA. When ORFB was deleted from the cDNA, this cDNA reduced the virulence when transformed into *C. parasitica*. In similar tests, the deletion of ORFA led to loss of the hypovirulence-associated traits (slow growth, low sporulation, etc.). Thus, it seems that ORFB is necessary for the expression of hypovirulence, whereas ORFA encodes many of the associated traits which are potentially disadvantageous in a biocontrol strain, reducing its environmental fitness. It may be possible, therefore, to manipulate the cDNA *in vitro* so that it has only the most desirable traits for biocontrol. Perhaps the most significant point, however, was that cDNA, when transformed into *C. parasitica* (causing the fungus to be hypovirulent), became stably integrated in the chromosomal genome, was maintained throughout the life cycle, even entering the ascospores. This would mean that biocontrol strains could be produced with permanent, stable hypovirulence (Deacon 1997).

Field control of chestnut blight

The possibility of controlling chestnut blight, following observations made in the field on the behaviour of hypovirulent strains of *Cryphonectria parasitica*, was realised immediately. The strategy was clear, however there where some obstacles, such as vegetative incompatibility. In other words, in a forest plot a randomly chosen hypovirulent strain is not able to cure all cankers (Grente & Sauret 1969). On the other hand the inoculation of active cankers with hypovirulent strains heals the cankers when strains with

the same vegetative compatibility (v-c) type are used (Bazzigher 1964, Grente & Sauret 1969).

Vegetative compatibility. Transmission of hypovirulence in *C. parasitica* is restricted by vegetative incompatibility, as shown both *in vitro* (Anagnostakis & Day 1979) and *in vivo* (Anagnostakis & Waggoner 1981). High vegetative compatibility group (VCG) diversity is thought to be the main reason for poor dissemination of hypovirulent strains in North America (Anagnostakis 1987). Viable anastomoses form only between strains which have identical alleles at all the v-c loci (vic-genes). The method devised by Anagnostakis (1988) for detecting vegetative compatibility *in vitro* is as follows. Small pieces (cubes less than 3 mm sides) of the mycelia to be tested are transferred from the edge of rapidly growing cultures on PDA, placed on new PDA not more than 5 mm apart, and incubated at 25-27°C in the dark for 4 days. At the end of this time mycelia that are vegetatively compatible have grown together, forming confluent mycelium. Incompatible strains grow to a meeting point in the agar and their mycelia remain separated by a 'barrage' line composed of dead hyphae with no covering aerial mycelium (Figures 1 and 2). If the plates are then exposed to light (16 h per day white fluorescent light, 25-27°C for 2 days) pycnidia form along the two sides of the barrage, making it more obvious.

When hypovirulent strains were introduced into the USA from Europe to hold chestnut blight in check, they gave only partial and local control. Apart from the specific differences of the host (*Castanea dentata* vs *Castanea sativa*), the pathogen population in the USA consists of numerous vegetative compatibility groups (VCGs), limiting the transfer of dsRNA because of cytoplasmic death during anastomosis. In Connecticut alone 67 v-c types were identified, and in the same state and in West Vir-

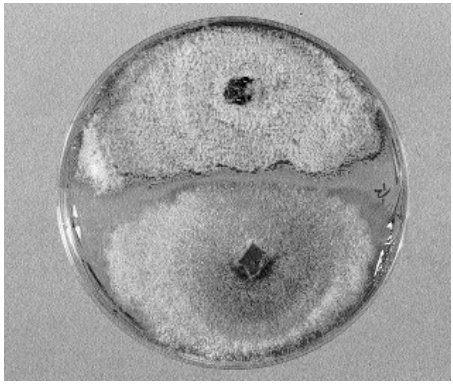

Figure 1.

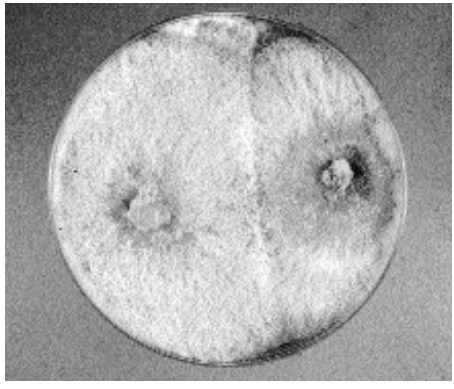

Figure 2.

Vegetative compatibility (Figure 1) and incompatibility (Figure 2) in strains of *Cryphonectria parasitica.*

ginia up to 27 and 48 different v-c types, respectively, were reported in single, small clear-cut plots (Anagnostakis & Kranz 1987, Milgroom et al. 1991). In addition in North America VCGs seem to be in a continuous state of flux: samplings of identical trees over several years showed that some of the predominant VCGs declined while new ones arose (Anagnostakis 1982). In contrast, the same dominant v-c types were found in the Switzerland (Canton Ticino) in 1976 (Bazzigher et al. 1981) and again in 1990 (Bissegger & Heiniger 1992).

The European population of *C. parasitica* is much more uniform than that in the USA, accounting for the success of biocontrol in Europe. Here the number of VCGs is quite low for specific regions or sites (Pennisi et al. 1992, Zambonelli & Zechini d'Aulerio 1986). The VCG diversity in a single stand is usually very limited, even as low as one per site and most isolates can often be assigned to one or two dominant VCGs (Bazzigher et al. 1981, Granata et al. 1992, Pennisi et al. 1992). Several infection sites with only one VCG are found at the forefront of the blight epidemic (Bissegger and Heiniger 1991, Xenopoulos 1981). In addition to low VCG diversity, spread of viral dsRNA in Europe is possibly further facilitated by hypovirulent isolates with broad conversion capacity (Bazzigher et al. 1981, Turchetti & Maresi 1988) capable of converting virulent strains from different VCGs (Kuhlman et al. 1984).

Sexual recombination of different v-c genotypes is the main source of VCG diversity in *C. parasitica* (Anagnostakis 1988). Perithecia are present in several European countries, although they are not very frequent in most areas. Their presence however is not critical since the sexual stage can also be produced by self-fertilisation (Milgroom et al. 1993, Puhalla & Anagnostakis 1971). Limited sexual reproduction appears to have contributed to the low v-c group diversity in Europe.

Operational strategies. Grente and collaborators started by treating some twelve chestnut orchards in southern France. Field applications were then carried out in other regions and results were so promising that a biocontrol program, supported by the Ministry of Agriculture, was established to assist chestnut growers (Grente 1965, Grente & Sauret 1978). Following VCG identification of the specific *C. parasitica*, the right mixtures of hypovirulent strains are prepared, packed in tubes, and distributed to chestnut growers. At the edge of the cankers, thin layer of bark are removed before injecting the hypovirulent mixture into holes (2-3 cm apart) around the cankers (Grente & Sauret 1978). Mixtures of hypovirulent strains, adapted to the different chestnut-growing regions, are available commercially.

A similar approach was followed in Italy and the work carried out by researchers of the universities of Milan and Udine is summarised below Along with the treatment of chestnut trees in the field, extensive studies on the spread of the disease and its natural recovery, the isolation of causal agents and hypovirulent strains, and

the study of compatibility biotypes, were undertaken (Cortesi & Milgroom 1998, Cortesi et al., 1996, Milgroom & Cortesi 1999). The procedure followed (Intropido et al. 1987, Bisiach et al. 1991) involves a sequence of approaches which can be summarised as follows. To begin with the site chosen for field applications is subdivided into areas that are homogenous for altitude, orientation, characteristics of the crop, etc. A census is then carried out by counting and classifying the different cankers present. Following pathogen isolation, the strains are checked for their relative vegetative compatibility *in vitro*. Suitable individual isolates or appropriate mixtures are grown on laboratory media or, in the case of large scale applications, in pilot fermenters (200 litres) as liquid cultures.

In small areas, disks of mycelium are inserted into holes cut in the bark around the border between canker infected and healthy tissues, each at the distance of a few centimetres. For large scale applications the mycelium, grown in fermenters, is packed in tooth-paste like tubes and can by applied to a larger number of plants even by unspecialized operators, following simple instructions. Usually some 20-30 cankers per hectare are inoculated. Obviously higher numbers of inoculations have the advantage of accelerating the healing process. This is not always possible in large areas and when tall trees are present, however natural spreading of hypovirulent strains can be achieved in a few years if the diffusion foci are carefully chosen.

The procedure has been tested in several Italian regions (Intropido et al. 1987, Bisiach et al. 1991). For example in Val Seriana in the province of Bergamo more than two-hundred cankers in forest and coppice stands were treated with a mixture of five hypovirulent strains that were compatible with the local population of the pathogen (Gobbi & Intropido 1984). Within six years most cankers had either healed or were in the process of doing so. At the same time in the treated lots the percentage of untreated active cankers decreased from 41 to 25%, indicating a natural spread of hypovirulence (Bisiach et al. 1988, 1991). In Friuli the work was focused on plants in the Natisone valleys (Grimacco - Udine), Mount Calvario (Gorizia) and the province of Pordenone.

Research on chestnut blight in Friuli. The work initiated in Milan was followed up in Udine university. In the following pages research work carried out in Friuli is presented.

Materials and methods. In addition to the isolates used in previous investigations carried out in the university of Milan, new strains were isolated from abroad and from various locations in the Friuli provinces. The methodology employed was the same as that tested and standardised in previous work.

Results

Population analysis. The obvious first step in the research was to analyse the population of *Cryphonectria parasitica*

strains present in the chestnut stands of the Friuli region. A survey of the cankers found in the provinces of Gorizia, Pordenone and Udine was carried out. At the same time a number of virulent and hypovirulent strains were isolated and characterised for eventual use in biological control (Gobbi & Locci 1987, 1989). dsRNA regulation of specific genes involved in mating types was investigated in the isolates obtained (Carpanelli et al. 1995). Strains isolated could be ascribed to the classical groupings of the fungus, that is normal strains with the typical morphology of the species (orange-yellow colony pigmentation, and good production of pycnidia) and altered ones, characterised by reduced pigmentation and poor pycnidium production *in vitro*. The relationships between type of canker and isolate characteristics were confirmed and heteroauxesis phenomena were found in normal strains only. In addition multiple infections were detected.

Pathogenicity tests confirmed the virulence of normal strains and the wide heterogeneity of the hypovirulent ones. Strains characterised by a large degree of compatibility and conversion capability were also identified.

Heteroauxesis. The phenomenon, consisting of colony malformations, was first discovered in strain VIR 1 (Gobbi et al. 1985). When subcultured, colonies of the virulent isolates of *Cryphonectria parasitica* became partly or completely distorted, with sectors and indentations of the margin. Mycelium growth was irregularly blocked producing a fan-like development of the advancing hyphae and chlamydospores. The dynamics of heteroauxesis were followed by monitoring the characteristics of the progeny obtained both from hyphal apices and conidia. The data indicated the possible cytoplasmic origin of the phenomenon. No correlation was found between heteroauxesis and pathogenicity, all isolates, no matter their colonial morphology, maintained their typical virulence.

The phenomenon was also investigated (Gobbi et al. 1989) by light and scanning electron microscopy (SEM). Heteroauxonic colonies show a clear-cut imbalance between vegetative and aerial mycelium, the former being favoured. The colony border is irregularly indented due to alterations in the hyphal branching pattern. Colonies are sectored because growth is not synchronised and the hyphal architecture does not depend on that of adjacent growth zones. Inside the sectors aerial growth is limited or almost absent. Vegetative hyphae branch abundantly with a rather lax pattern, resulting in irregularities of the colony margin. Under SEM the arrested hyphae are clearly distinguished by the enlargements of their tips. They show distortions of their linear development in addition to lysis and extrusion of cytoplasmic material which accumulates at the tip, a phenomenon similar to the 'balloon' syndrome observed in *Neurospora crassa* (Bainbridge et al. 1979).

Mitochondrial DNA polymorphism. As it is not transferred by anastomo-

sis, mitochondrial DNA (mtDNA) was chosen as a stable natural marker for epidemiological studies and for monitoring *C. parasitica* in natural environments (Gobbi & Locci 1988, 1995). The size of mtDNA varies in the different strains from 137.3 to 146 kb, a value larger than that of other fungi. The mtDNA has a marked polymorphism of the restriction fragment length (RFLP) and could be used as a source of genetic markers. With this in mind a methodology for mapping the polymorphic sites in the mtDNA molecule was devised (Gobbi et al. 1991). A tentative physical map of strain Ep155, showing a large sized mtDNA molecule, was constructed with the aid of restriction endonucleases (KpnI, SacI and Eco RI) by developing appropriate clonation and hybridisation strategies.

In more recent studies (Gobbi et al. 2002a, 2003) known mtDNA polymorphisms were mapped and found to cluster in four regions of the mtDNA molecule, particularly in the RFLP region 2 where five mtDNA haplotypes out of 13 strains were identified. The region included an area of 8.4 kbp which was entirely sequenced in strain Ep155 showing the presence of two introns. An internal 3.2 kbp portion was sequenced also in six additional strains. Subsequent comparison of the *C. parasitica* mitochondrial intronic ORFs revealed similarities to known endonucleases such as those of *Podospora anserina* and *Neurospora crassa*. DNA sequence analysis revealed that three polymorphisms of this mtDNA region within the population of 12 strains were due to the optional presence in the *ND5* gene of an intron and an intervening sequence within the intron.

The pUG1 plasmid. The involvement of plasmids as agents of heteroauxesis was suggested when the phenomenon was first observed; targeted research confirmed the hypothesis. A circular plasmid, pUG1, was finally isolated and characterised (Gobbi & Locci 1990; Gobbi et al. 1996, 1997a, 1997b, 2002b, 2002c; Rekab et al. 2001).

Plasmids are common extra-chromosomal DNA molecules that can reproduce independently from chromosomal DNA. In filamentous fungi they are generally localised in mitochondria and are of the linear type (Nargang 1985). Most circular mitochondrial plasmids are derived from mtDNA and have sequence homology with the host nucleic acids. However, a minority, the 'true plasmids', have their own independent sequence (Griffiths 1995). The *Cryphonectria parasitica* pUG1 plasmid belongs to this group and shows a high degree of similarity to the Fiji and LaBelle plasmids of *Neurospora intermedia*. These three plasmids all encode a particular family B DNA polymerase which is characterised by a specific signature, TTD instead of DTD, in the motif C typical of this family. Moreover, they are closely related to the linear plasmid polymerases (Gobbi et al. 1997a). These unique features suggest that plasmids pUG1, Fiji and LaBelle constitute a new subgroup of circular mitochondrial plasmids and that they may share a common origin.

Plasmids of pathogenic fungi and pathogenicity are generally considered to be correlated (Griffiths 1995). In *C. parasitica*, plasmid pUG1 was initially found in strains showing a senescent phenotype called heteroauxesis. Since then it has been hypothesised that the plasmid might reduce the fitness of the strain and consequently lower pathogenicity. A second *C. parasitica* plasmid, named pCRY, with 99.8% nucleotide sequence identity to pUG1, has recently been reported (Monteiro-Vittorello et al. 2000). pCRY has been proven to affect the virulence of at least one strain of *C. parasitica*. Amplification by specific PCR of some 200 strains showed the presence of pUG1-like plasmids in 22% of the population examined. The entire plasmid molecules were amplified by multiplex PCR and the products showed different RFLP patterns. The variability was mostly in a non-coding region of the molecule that had been sequenced in some representative strains, enabling the evolution of the molecule to be elucidated. The data obtained show that mitochondrial plasmids of *C. parasitica* are an almost homogeneous family (designated pCp) that can be divided into two clusters based on the presence/absence respectively of a 60 nucleotide region in North American and European plasmids.

Conclusion. The epidemic of chestnut blight hit European chestnut stands with a violence equal to that observed in North America. At the time there was little doubt about the fate of the trees. Instead just a decade and a half later they started recovering spontaneously and the phenomenon led to the successful control of the disease. Natural hypovirulence alone could not bring about such results, or at least not in such a relatively short time, without human aid. In nature the dissemination of hypovirulent strains is not favoured over the virulent ones (Anagnostakis 1987, Heiniger & Rigling 1994), because of the biological characteristics of the former (growth rate, sporulation, etc.). This is why the artificial release of hypovirulent strains has been fundamental in tilting the balance in favour of hypovirulence.

However, as shown above, recovery was by no means immediate and automatic, but required extensive research efforts to overcome a series of obstacles (selection of proper strains, vegetative compatibility, etc.). There are still problems but these may be solved by improving hypovirulent strains with the view of obtaining individuals with stable, permanent hypovirulence. Characters associated with hypovirulence do not always favour ecological fitness and the spread in nature of these strains. There is in this regard the interesting possibility of manipulating the ORF regions of the dsRNA involved without interfering with hypovirulence. In any case the control of the disease has so far been a success.

Finally, as stressed by Heiniger and Rigling (1994), the relevance of the phenomenon of hypovirulence in long-lived plants, potentially achievable with a single application, is of great biological interest.

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