Biological Control of Chestnut Blight in France

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ABSTRACT.— A brief history of the discovery of hypovirulent forms of *Endothia parasitica* is presented. The properties of hypovirulence are discussed, including the exclusion of diseased bark, transmissibility and vegetative incompatibility. A summary is presented describing the practical use of the hypovirulence phenomenon to biologically control chestnut blight in France.

INITIAL DEVELOPMENTS IN METHOD

In 1951, in the Genoa region of Italy, A. Biraghi, a phytopathologist at the Forestry Institute of Florence, observed for the first time the spontaneous healing of cankers on sprouts growing from the stumps of chestnut trees. Between 1920 and 1950 *Endothia parasitica* (Murr.) P. J. & H. W. And. caused extensive damage in Italy. The fruit-bearing trees that had been cultivated to full height died, and only the stumps remained alive. When the dead trees were cut, the stumps sprouted, establishing new coppice sprouts. A few years later the sprouts were killed by cankers. The trees were again cut and subsequent sprouts killed by *E. parasitica.* The phenomenon observed by Biraghi occurred after three or four successive growth cycles of these coppices.

In the case of the cankers that were healing spontaneously, it was evident that the tree had formed a new healthy bark underneath the cankerous bark and the parasite had not penetrated the new bark. Biraghi's initial observations revealed the spontaneous healing of only a few cankers, either on the same sprout or on the same stump, while other cankers continued to spread. Instead of concluding that the fungus had lost its virulence, Biraghi thought that the host plant had developed a partial resistance after being cut several times. This conclusion had no scientific basis and was considered unsatisfactory in scientific circles. Consequently, a group of experts made up of Italian, Spanish, Swiss, and French phytopathologists was sent by the Food and Agriculture Organization to study the problem.

In 1964, the phenomenon of spontaneous healing had become widespread throughout the Genoa region and in part of the Piedmont chestnut plantation (in the Bergamo and Cuneo regions). In many plantations the cankers had ceased to spread at all. The obvious conclusion was that the disease had completely disappeared from most plantations. Pathologists then evaluated the practical use of this phenomenon.

STUDIES OF SPONTANEOUS HEALING

The white strains

The French school had suggested that the healing may have been caused by some change in the parasite. Initially we cultured the fungi present in cankers and compared isolates obtained from cankers that were spreading, healing, and those that had healed completely. Numerous isolations of each canker of each type were carried out in the laboratory.

In the case of spreading cankers, all the samples showed the presence of the so-called "normal" form of the fungal parasite. Under the conditions used for these cultures, the characteristics of the fungus are a white or colorless mycelium, forming orange stromas in places. These stromas produce pycnidia, the asexual fruiting organs of the parasite. When the culture is periodically subjected to light, the pycnidia form concentric circles. These circles correspond to the edge of the thallus each time light is applied.

In the case of cankers that were in the process of healing, a new type of culture, known as a "B" or "white" culture, was obtained alongside the normal cultures, known as "N". The new type B did not produce pycnidia under conditions in which these structures were produced by the N types. The percentage of B types present correlated directly with the degree to which the canker healed, and varied from 5 percent to 100 percent, depending on the situation. In the case of cankers which had healed completely, a high percentage of the samples (10 to 20 percent) either did not yield *E.* parasitica or were type B cultures. It was then concluded that healing statistically correlated with the presence of parasite cultures of the white variety. The next step was to show that there was a cause and effect relationship, that is, a biological correlation, between the two phenomena.

"B" STRAIN PROPERTIES

Hypovirulence

When a tree is inoculated with a B strain, a small canker develops over a limited area. The fungus produces a red band, about 1 cm in diameter, around the inoculation site before ceasing to spread. Callus then forms at the inoculation site. When a cross-section is cut through the canker it becomes apparent that under the diseased bark there is a new bark, formed by a suberophellodermal generative layer in reaction to the infection.

It is interesting to compare this with what happens in the case of inoculation with a normal N strain. The initial stage of development is identical. *Endothia* forms a layer of mycelium parallel to the surface of the bark. The plant forms a suberophellodermal generative layer underneath the infected bark. In the case of a B strain, this area is continuous and suberization is rapid. The opposite is true of an N strain: the generative area is limited and suberization is slow. Consequently, when the B strain is used, the cork barrier formed by the suberophellodermal area arrests the spread of the mycelium. In the case of the N strain, the mycelium has time to cross the cork barrier being formed by the plant in reaction to the infection. After several months a canker is formed by alternating layers of mycelium, diseased tissue and cork layer. The white strains, then, have lost the ability to combat the natural defenses of the plant. They have not lost all their virulence, simply a part of it. We have termed them "hypovirulent."

The ability to exclude

Hypovirulence explains the healing of cankers produced by inoculation of a B strain, but it does not explain satisfactorily the healing of cankers which were already developing. Experiments have shown that the arrival of a B strain after the development of an N strain can bring about healing.

The trunk of a susceptible tree, 15-20 cm in diameter and covered with smooth bark, was inoculated with an N strain. The standard method was used (a pellet of mycelium placed in a wound 7 mm in diameter). The canker was left to spread for eight months and, at the end of this period, a clearly defined lesion, approximately 15 cm long and of a similar width, was evident. Wounds were then made with a cork-borer in the uninfected bark all along the edge of the lesion. The wounds were approximately 2 cm apart, and were bored adjacent to the edge of the lesion. A pellet of mycelium of a hypovirulent B strain was placed in each wound. It was found that the canker stopped spreading and, after several months, calluses began to appear all along the edge, indicating that healing had begun. A year later it was seen that a new bark, uninfected by the fungus, had formed under the lesion, separating it from the healthy tissues beneath. The infected bark dried out and, in some cases, was even rejected in the form of decaying black scales. We have called this characteristic rejection of the diseased bark by the hypovirulent strains "exclusion." Hypovirulence can be termed "exclusive." The B strains are "exclusive hypovirulents. "

The "transmissible" nature of hypovirulence

If, in the exclusion experiment, one tries to cultivate the parasite by taking sample fragments of bark and making laboratory cultures, it is found that, one to two months after peripheral inoculation of the B strain, the fragments taken from the edge of the lesion show type B cultures only. Those taken from inside the margin of the lesion, that is, from the bark infected by the N strain, show a high percentage (30 to 80 percent) of type B cultures. Those taken nearer the center of the lesion show a proportionately smaller percentage of B cultures, the nearer they are to the center. Following the same procedure five to six months later, the percentages of B cultures are higher and have the same proportional distribution.

It is particularly surprising to find that, in places where the N strain was present before peripheral inoculation, the B strain mycelium is present after inoculation. We concluded that the N strain must have been changed to the B strain. This transformation can, in fact, be brought about *in vitro* by the combined culture of the two strains N and B. When the filaments from N and B come in contact, there is a period of variable growth, then subsequent filaments continue to grow as form B. The further the thalli are from each other, the longer they grow as N and B. One very important point is that if the mycelium that developed initially as an N form is subcultured, the resulting cultures are of type B, indicating a transformation from type N to type B. In other words, the B type is "transmissible." It also can be shown that the cultures obtained as a result of this transformation are genuinely hypovirulent and exclusive. This ability to transform by transmission explains the phenomenon of exclusion.

Numerous laboratory experiments have shown that the transformation of the N thallus by the B thallus follows anastomosis, in other words, a union between mycelial filaments of the two strains. This anastomosis allows the exchange of nuclear and cytoplasmic material between the two thalli. It seems reasonable to assume that in the course of this exchange, the cytological determinants of hypovirulence pass from one strain to the other.

Vegetative incompatibility

When several B strains and several N strains are paired, it is found that transformation does not take place in every pair. It also is found that, when the transformation does not take place, anastomosis causes degeneration of the protoplasm at the point where the two strains meet. This phenomenon has been called vegetative incompatibility, and is particularly significant when it comes to the application of biological control.

APPLICATION TO BIOLOGICAL CONTROL

General principles

Once it had been demonstrated that spontaneous healing of the cankers resulted from the occurrence of strains manifesting "exclusive transmissible hypovirulence," it became clear that this phenomenon had spread over vast areas of Italy, in some cases totally eliminating the disease. It also became possible to envisage its application in areas where hypovirulent strains had not yet occurred or were still rare. One such area was France.

The principle behind the plan of action was as follows: since the hypovirulent strains which occurred spontaneously had spread without human intervention and had succeeded in checking the disease in Italy after 20 to 30 years, it should be possible to speed up the process by introducing them artificially in chestnut stands. If hypovirulence is a kind of contagious disease affecting the parasite, it should be feasible to control *Endothia* by spreading hypovirulence among the parasite population. In France we have experimented with the inoculation of B strains onto cankers, using the same process adopted for the exclusion experiments.

In practice, a canker is inoculated in each infection center of the disease. On French chestnut plantations there can be 5 to 20 centers per hectare, and each center can include 1 to 20 cankers, sometimes more. Some cankers grow on high branches, in which case the treatment is not applied. When experimenting, an average of 10 cankers per hectare is treated. In the years that follow, the results are assessed. If necessary, treatment is applied to any new cankers which have appeared since the last application or to those which have not responded satisfactorily. It has been found that hypovirulent strains introduced in this way spread without any assistance, and that, after 3 to 5 years, this dissemination can become apparent in a radius of 5 to 10 meters around the introduction site. After 10 years the stand can be completely healed.

Dissemination of natural white strains

The isolations carried out in the Italian plantations of the Piedmont in 1972, allowed us to calculate the percentage of white strains in relation to normal strains. On average, this was between 20 percent and 30 percent. In 1977, Mario Palenzona, of the National Institute of Woody Plants in Torino, studied a large number of cankers in the same region. This was part of the Institute's program of scientific collaboration with our laboratory. The results of this study showed that a very high proportion of cankers (80 percent) are in the process of healing. In 1977, the proportion of B strains in the isolations was between 60 percent and 90 percent. It is clear, then, that there has been progress since our initial studies, and that the hypovirulent strains have spread without human aid.

The healing of treated cankers

Biological control works on the principle that the pathogen can be changed in such a way as to make it hypovirulent. The inoculation of developing cankers with hypovirulent strains is carried out with the aim of transforming the entire parasite population. In the short term, this operation results in the healing of the inoculated cankers. This is beneficial to the plant itself, especially if all the cankers on the branches of the infected tree can be treated. It is valid to say that the application of biological control has two results: the healing of the diseased tree, and the arresting of the parasite. Whether one is concerned with cultivating the chestnut tree for its fruit or for reforestation purposes, the first result is certainly important. However, the second result far outweighs the first in importance.

The dissemination of artifically introduced hypovirulent strains

In Italy, the spontaneous hypovirulent strains spread naturally throughout the *Endothia* zone, causing a general regression of the disease. This process took several decades (between 30 and 50 years). In theory, the dissemination of hypovirulent strains by artificial means and at a higher density (at least ten places per hectare) should take place far more quickly.

Experiments carried out in France since 1966 support such a supposition. In the locality of Mayons, an area in the mountainous region of the Maures, plants were treated from 1967 to 1972. After three years, 50 percent of the inoculated cankers were found to have healed. After five years, the percentage was 70 percent. Even during the first years after treatment it was possible to observe the healing of untreated cankers close to the sites where the hypovirulent strain had been introduced. From the fifth year onward, dissemination was complete within a radius of approximately five meters around the treated cankers. Ten years later, there were no more cankers developing on the treated plantations.

Strain compatibility

In 1968 we treated a large number of trees in a region near Mayons: the locality of Gonfaron. Three years later, only 20 percent of the cankers were in the process of healing; less than in the region of Mayons. In order to determine the cause of this relative failure, we tried to determine whether or not the virulent strains present in Gonfaron were capable of being transformed by the inoculated B strain. Laboratory tests revealed incompatibility. A compatible B strain was selected, and treatments carried out after 1971 produced satisfactory results.

This study served to highlight the importance of testing strain compatibility and selecting B strains before treatment. However, it is worth noting that, even with a B strain which proves incompatible in laboratory testing, a 20 percent success rate can be obtained. The reisolation of the B strain from the cankers which have healed produces a new hypovirulent strain, compatible with all the N strains in the same locality. From theoretical studies on hypovirulence, this is exactly what one would expect. It is also worth remembering that, on the tree itself, compatibility between B and N strains does not operate with the same rigidity as in laboratory tests.

Natural occurrence of hypovirulent strains

The phenomenon observed in Italy was caused by the appearance of a new type of strain. It would be interesting to know whether the same thing has occurred elsewhere. As early as 1959, a survey carried out in France showed that in those areas longest affected by *Endothia,* hypovirulent strains were, in fact, present in very few places. It appears that hypovirulent strains now also occur in the U.S.A. The opinion is sometimes expressed that mutation towards hypovirulence is inevitable, and the scientific thinking behind this opinion makes an interesting topic for discussion.

THE APPLICATION OF BIOLOGICAL CONTROL

Experimentation

Between 1966 and 1974 the method of application was tested over limited areas (approximately 500 hectares) on the chestnut plantations of the Maures Range. Between 1972 and 1974 tests were also carried out over smaller areas in the Cevennes region. Since 1974 this method has been applied far more extensively, and the Ministry of Agriculture has provided the necessary financial backing for the treatment of 18,000 hectares throughout France over a period of four years.

Organization of the control program

Technical Procedures

The work falls into several stages:

1. Surveying and mapping of the infected plantations;
2. Selection of areas to be treated;
3. Sampling in each locality, in order to isolate the virulent strain present;
4. Laboratory selection of hypovirulent strains compatible with the virulent strains;
5. Production of the inoculum;
6. Education of farmers and demonstration of treatment techniques;
7. Treatment by farmers;
8. Supervision of treatment;
9. Assessment of results and remedial studies in cases of failure.

Rather than giving a detailed explanation of every stage, we shall make a few important points.

The selection of compatible strains

When a pure culture of the virulent strain to be controlled is available, the tests to be carried out in the laboratory are those for transformation by anastomosis, as detailed above.

When the laboratory has a collection of approximately 30 hypovirulent strains, the following procedure should be adopted: each virulent strain is confronted by the hypovirulent strains, and, in 95 percent of all cases, a compatible strain emerges from this simple test (90,000 tests are carried out each year). However, in certain cases, this method is not successful, and the method known as forced anastomosis is then applied. This consists of multiplying the number of confrontations between two incompatible strains. Generally, 2 to 3 percent of these confrontations result in a transformation from **N** to B. The N strain which has been changed to B is then reisolated, and this new strain is sure to be compatible with the N strain, and with all strains having the same nuclear characteristics. When this last method fails to produce the desired result (0.5 percent of all cases), the same confrontation procedures are carried out on the tree itself, and it is almost certain that a compatible B strain will be obtained.

The inoculum

After several years of study aimed at perfecting a testing technique for the genetic resistance of several *Castanea* hybrids, Bazzigher' s method has now been adopted. This method was described in 1963 as "schuttel kulter" or mobile culture, and it produces an inoculum in the form of "pellets." Bazzigher has pointed out the advantages of this form of inoculum; the only one to ensure an inoculation success rate of 100 percent.

We have found this to be the best type of inoculum for the inoculation of hypovirulent strains. We prefer it to all the other types, even though its use entails far more work. In particular, when inoculating the cankers, a line of wounds has to be made all around the canker and a pellet of inoculum placed in each hole. This is difficult when working on trees in natural conditions. It would be easier to use mycelium cultures on a semi-liquid nutritive medium, and to apply this with a syringe or dispensing instrument. Nevertheless, we prefer the pellet method.

The inoculation wounds have to be made in the healthy part of the bark and be tangential to the edge of the lesion. To prevent the inoculum from drying out, the hole is sealed with a strip of masking tape (this need only stay in place for 24 hours).

Production of the inoculum

During the early years, the pellets were produced in glass bottles placed on a "Roller-type" agitator. This technique is extremely laborious and expensive. After several years of research, a method of producing pellets in fermenters was perfected. The workings of the set of six fermenters, each with a capacity of 50 1, are highly complex. They have been made completely automatic with the aid of a microcomputer, so that in four months it is possible to prepare 2,000 bottles, each containing 150 ml of inoculum. This provides enough inoculum to treat between 18,000 and 20,000 hectares.

General organization of the "Biological Control Division"

It is anticipated that 18,000 hectares will be treated over a period of four successive years. It has been necessary to form a team of specialists that were able to apply the method perfected by us in our work for the National Institute for Agronomic Research. The division is made up of two subdivisions.

1. A fieldwork division, responsible for coordinating the activities of eight to ten technicians, who

carry out surveys, organize treatment of the different chestnut-growing regions, and supervise the work;

2. A laboratory where virulent strains are isolated, hypovirulent strains selected, and the inoculum produced. It employs nine people.

Finance

Given that an area of 18,000 hectares is to be treated, the Biological Control Division costs 1,500,000 F ($300,000) per annum to run. In addition, there was an initial outlay of 500,000 F ($100,000). One must also take into account the work of the farmers who carry out the treatment, working at an average rate of 10 minutes per tree, or 100 minutes per hectare. This information is merely intended as a guide.

CONCLUSION

The scientific analysis of a natural phenomenon has led to its practical application in the field of biological control. This control exploits the natural defense mechanisms of the plant and the natural variability of the parasite. The application of similar methods to other pathogens would be highly desirable, but it would require a more detailed knowledge of the cellular mechanisms of hypovirulence, and this, in turn, would call for international cooperation.