Research Carried Out in France Into Diseases of the Chestnut Tree

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ABSTRACT.—The root rot of European chestnut caused by *Phytophthora cinnamomi* is described. The nature of the infection process, possible resistance mechanisms, and control by use of mycorrhizae are topics that have been investigated.

The cultural features of normal and hypovirulent isolates of *Endothia parasitica* are contrasted. Vegetative segregation of hypovirulent B forms into N, B and JR types and a description of the infective properties of B and JR are covered. The role of diaporthine, a toxin produced primarily by virulent strains of *E. parasitica,* is discussed in relation to the host-parasite interaction. The paper concludes by exploring the development of other biological control systems based on hypovirulence.

RESEARCH INTO ROOT ROT CAUSED BY *PHYTOPHTHORA*

The root rot of chestnut caused by *Phytophthora cinnamomi* Rands was very severe in France at the beginning of this century, resulting in the death of more than 50 percent of the trees. Because of this disease, chestnut farming was abandoned in most of the central and southern mountainous regions of France. This seriously affected soil protection and water distribution, because chestnut was the only hardwood tree able to cover the arid slopes of these granitic and schistose mountains (alt. 700-900 m). Chestnut was replaced by softwood trees that increased the risk of forest fires. Finally, the nut crop provided the population both with a source of nutrition and the possibility of a yearly financial return. Replacement of the chestnut by softwood trees sped up the flow of population away from these areas to the richer valleys.

The cyclic progress of the disease consists of alternating crises and remissions. The disease most often develops in cool, fertile soil well suited to fruit-growing. High mortality occurs in dry years. Once the root hairs are killed by the parasite, the trees are no longer able to cope with water stress. During humid periods the trees do not die, but the fungus spreads, harming all the roots. The disease progresses without having any visible effects; the tree dies when the entire root system is diseased. A continuous struggle takes place between *Phytophthora,* as it destroys the root hairs, and the persistent rhizogenesis of the tree. This equilibrium can only be broken after many years, sometimes 10, sometimes as many as 20.

The physiology of the disease has been studied in the laboratory in an effort to perfect testing methods for the selection of resistant ecotypes of *Castanea sativa* Mill. and of numerous simple or complex hybrids of the species *C. sativa, C. crenata* Sieb. & Zucc. and *C. mollisimma* Bl. For many years, wounds in the main root have been injected using mycelium as inoculum. The results have not been consistent. Now that it has been established that infection begins in the root hair, a technique of soaking the roots in a semi-liquid suspension of microthalli of the fungus has also

been adopted. The results are more predictable, but the post-inoculation development of the lesions is still uncertain.

It has been possible to relate the progress of the disease to the physiology of the plant. Extended studies have shown that:

a) If a young chestnut tree (1-3 years old) is inoculated during dormancy, lesions appear on the roots and death follows quickly, regardless of the genotype of the plant. The inoculation must be made at a temperature of at least 16-17 C.

b) When inoculation is carried out during the active growth period, no lesions develop, regardless of the genotype of the plant.

c) If a comparison is made between types with known practical resistance (trees which have resisted for several years in naturally contaminated environments) and sensitive types, no difference in the results of inoculation is found, whether this was carried out during dormancy or during active growth.

When studying the development of lesions on the roots, it has generally been found that, under natural conditions, no infection occurs on the roots during dormancy, because the temperature is too low. Whenever the soil is warmed during the winter, the fungus is able to become active again and produce lesions on the dormant roots. In the case of sensitive types, these lesions will continue to develop during the active growing period. The roots of resistant types, however, react by separating diseased parts of the root from the healthy ones. This elimination comes from the formation of a ring of cork and of an abcission zone. It seems then, that resistance is the ability to reject lesions which have developed during dormancy and that this rejection takes place during the active growth period, by a process of active defense.

The development of mycorrhizal studies, carried out at the I.N.R.A.'s Station in Clermont-Ferrand, has opened a new line of research into control methods. It has been found that mycorrhizal populations associated with the roots vary greatly, depending on the environment. Young plants (3-5 years) that are not killed by the parasite have a very different mycorrhizal flora from those that die. Work is in progress to try to isolate the associated mycorrhizal symbionts.

Tests carried out *in vitro* have shown that several mycorrhizal or peritrophic fungi are able to combat *Phytophthora cinnamomi* very effectively. Following from Marx and numerous American researchers who have shown that certain mycorrhizal fungi can protect *Pin us echinata* Mill. against *Phytophthora cinnamomi,* we have started researching the practical application of this natural antagonism. The use of mycorrhizal symbiosis is usually limited, because of the difficulties of cultivating the fungus on a "commercial" scale, and because it can be eliminated by competitive microorganisms in natural soils. Our team has perfected methods of multiplying the mycorrhizae by hydroponic culture on equipment known as "nutrient mist boxes." As a result of this work, our hopes for biological control have greatly increased.

RESEARCH INTO *ENDOTHIA PARASITICA*

Factors Determining Hypovirulence

After discovering "exclusive transmissible hypovirulence," we carried out research into the factors determining this phenomenon at the cellular level. The work focused on the study of single-spore progeny. Asexual spores, contained in pycnidia, were used.

The production of N-type spores is relatively easy on a Maltea-Moser agar. On this medium, the mycelium remains colorless (appearing white when it is aerial), only the pycnidia are colored with a yellow-orange pigment. These appear on the third day, an alternation of light and darkness provides a means of recording the growth of the cultures. The pycnidia appear at the point reached by the edge of the thallus, each time light is applied. A few hours of light are enough to initiate pycnidial formation. A series of concentric circles is obtained. In continuous darkness, the pycnidia are thinly distributed, and appear on mycelium after 7 days. In the case of B strains, the pycnidia only appear after 15 or 20 days, provided the culture is subjected to light for the greater part of the time (more than 50 percent). They are few in number, and show no particular distribution pattern.

Once the two types of asexual spores were obtained from pycnidia, a karyological study was carried out. The N-strain spores contain a single spherical nucleus, which occupies approximately one third of the spore volume. The B-strain nucleus is more difficult to stain, but appears to be single. The spore contains an element of large diameter which gives it a somewhat different appearance, being swollen in the middle. The nucleus takes up a smaller fraction of the total volume.

Vegetative Segregation

Single-spore progenies are obtained by classical laboratory techniques. From N-type cultures that have been isolated from natural cankers, the progeny are only of N type. No vegetative segregation occurs. As in the case of the first generation, the second shows no vegetative segregation. The same is true of all later generations. In some cases we have been able to carry the progenies through to the tenth generation.

The progeny of wild B-strain spores leads to an extremely complex vegetative segregation. Many different types of cultures can be identified. However, after one or two transfers, the morphology becomes stable, and three main types emerge. Two of these types are similar in every respect to the N and B types already known. The third type is very distinctive. The cultures show a mycelium with swollen, uneven, irregular cells. The orange

pigment is situated inside the cells. Numerous spores are produced by budding along the cell walls. On the Maltea medium, the N type only forms pigment in the cells of fruit-bearing stromata, and spores only appear in these stromata. The B type forms pigment and spores after approximately 20 days. The new type forms spores and pigment in a diffuse manner. We have termed this type JR. The JR types are not all identical. Numerous minor morphological differences can be identified, varying immediately after transfer but then stabilizing.

In addition to these three types, one sometimes finds cultures with colored mycelium that form pycnidia, cultures with large numbers of very small pycnidia, and cultures which grow unevenly, forming several sectors. Normally, these types are unstable and sometimes sublethal. Most of them change during transfer and eventually show the characteristics of one of the main types, N, B or JR.

Like the wild N types, the N's which have been produced by B segregation show no segregation. The B types behave exactly like wild B types, segregating into N, B and JR. The clearly defined JR types produce no vegetative segregation for several successive generations (more than 12 have been studied).

The pattern of vegetative segregation clearly conforms to a pattern of two nonsegregative types N and JR, and one segregative type B. It is also possible to obtain similar segregations with cultures of apex and fragments of thalli. The evidence seems to point clearly in the direction of extranuclear heredity.

As early as the second generation, all the thalli come from a single uninucleate spore, ruling out the possibility of heterokaryosis. The intervention of a parasexual cycle is equally impossible, therefore the cause of the variation must lie in some extranuclear mechanism.

Basically, there are three possible explanations for results, obtained from the experiments: 1) heredity of a cellular particle or organelle, existing in two forms capable of combining in a stable manner; 2) heredity of a self-maintained cellular mode of functioning, as with flux equilibriums; and, 3) heredity of viral particles. The last two hypotheses are difficult to reconcile with the existence of three types of characteristic vegetative segregation, or with the existence of other properties, such as virulence and "transmissibility," described later. Further complementary hypotheses would be necessary. In the case of the viral hypothesis, there would have to be several viruses with complex interactions. This is what the Connecticut team has just suggested.

Although it is far from proven, the first hypothesis seems the most attractive. It is difficult to resist the temptation of making an analogy with the "small colony" mutants in yeast (called rominus). These are mitochondrial mutants with deficiencies of the respiratory system.

Another analogy could be made with the "Poky" mutant of *Neurospora,* which is determined by similar factors. Perhaps the anomalous mitochondria carry an endomitochrondrial virus.

The virulence of these types obtained in the segregating of B types has been studied by inoculating *C. sativa.* The N types are just as virulent as the wild N types, so it is clear that vegetative segregation allows full restoration of virulence. The B and JR types, on the other hand, are hypovirulent, just like wild B types.

"Infectious Property"

It is known that, provided they are compatible, contact between a wild B type and a wild N type causes transformation of N to B as a result of the anastomosis formed between the two thalli. The B types always bring about a unilateral transformation of N types to B, that is, the N type only is transformed, taking the morphology of the B type. The most interesting experiment involves the anastomosis of N with JR. Both N and JR are found to be transformed to B, indicating bilateral transformation.

These facts have led us to construct a somewhat over-simplified hypothesis, but one which, nevertheless, accounts for all the facts observed.

It is possible that the N type contains a normal cytoplasmic determinant in its pure form and the JR type contains a mutated cytoplasmic determinant in its pure form. Finally, the B type may contain a mixture of the two cytoplasmic determinants. This would account for vegetative segregation and the results of anastomosis. The mutated cytoplasmic determinant would have to have greater powers of replication than the normal determinant. Such a hypothesis is perfectly plausible, since it corresponds to the so-called "suppressive" types in "small colony" yeast.

The "Heterokaryon" Test

Auxotrophic variants of B and N were obtained by U.V. irradiation. An auxotrophic N variant was anastomosed with a B prototroph on a complete medium. N was transformed to B but the mycelium transformed from N remained auxotrophic, even though it acquired B morphology. The prototroph had evidently transmitted its hypovirulence but not its auxotrophy. The reverse transformation (from a B prototroph to an N auxotroph) also showed the independent transmission of auxotrophic characteristics in relation to hypovirulence. It was also found that no heterokaryon was formed in these experiments. Hypovirulence, then, is totally independent of characteristics transmitted by the nucleus.

Mutagenesis experiments have been carried out, in an attempt to obtain mitochondrial mutants, using mutagens (acridine orange and acriflavine) known for their favorable action on mitochondria' DNA's and RNA's. A few mutants resembling B

types were obtained, but these types lack "contagious" properties.

To conclude this section, it should be said that further work on the factors determining hypovirulence at the cellular level would be particularly profitable. This work would include studies on sexual reproduction, mutagenesis, ultrastructure and, in particular, the biochemistry of B mutants. A study of the viral hypothesis also should prove fruitful. However, in our opinion, the virus would have to be fixed on an organelle occurring in small numbers within the cell, if an explanation was to be found for the percentages of segregation observed. The virus may be endomitochondrial.

Studies of hypovirulence at the level of host parasite relationships

Diaporthine

Endothia parasitica (Mum) P. J. and H. W. And. secretes toxins, one of which, "Diaporthine," has been studied by Bazzigher. In order to establish the role of diaporthine in infection, we have compared the production of toxin by N and B strains. At the same time, we have evaluated the infectious properties of the strains by measuring the speed of development of lesions on sensitive chestnut trees after inoculations. The inoculations were carried out according to very strict procedures.

There is a correlation between infectious properties and the production of diaporthine. It is also clear that the hypovirulent strains produce very little diaporthine. The role of this toxin at tissue level is interesting, and a study of this topic was carried out by F. Riou during a course of training at our station. Initially, the reactions of the plant were studied.

The first reaction to the infection involves the cells 1 mm from the edge of the lesion. After inoculation with a hypovirulent strain, the cellular membranes become thicker. Inoculation with a virulent strain does not give rise to this transformation of the membranes. If cross-sections are cut three months after inoculation with B strains, a complete perfectly suberized cork barrier is found. In the case of N strains, small islands of suberized cells appear, but they do not form a continuous barrier.

From this one can conclude that virulent strains hinder the formation of reactionary suberophellodermic zones.

The role of diaporthine in the prevention of generative zones has been studied by applying diluted solutions of diaporthine to bark wounds. The experiments were carried out using two different methods. In the first method, the hypovirulent strain was inoculated into a hole adjacent to the one used for the diaporthine. It was hoped that, in this way, the virulence lost by the B strains, would be restored. Unfortunately, the diaporthine was used in concentrations that inhibited the development of the fungus. In the second series of experiments, an N strain was inoculated into holes that had been used on all previous days for the application of diaporthine solution. We know, from Bazzigher's work, that wounds can only be infected during the first five days. Daily application of diaporthine prolongs the sensitivity of the wounds, as long as the toxin is applied. Our experiments have covered periods of up to 20 days. This would suggest that the toxin slows down or prevents wound healing. Further experiments will have to be carried out to establish whether or not this action affects infection by N strains, by slowing the suberization of the cells produced by the generative zones. Initial observations seem to indicate this.

The problems of vegetative compatibility

Vegetative compatibility was discovered in 1976 after inoculation experiments using several hypovirulent and virulent strains isolated in Italy and France. Previous experiments had shown time and again that the transformation from N to B was a result of anastomosis between mycelial filaments. Studies carried out under the microscope show that, in the case of compatible strains, the protoplasm of the filaments does not change. In the case of incompatible strains, the protoplasm of the cells degenerates and sometimes the walls break down, allowing the protoplasm to escape. Research is now being carried out in an attempt to establish whether the compatibility between N and B strains relates to the compatibility groups studied by Anagnostakis.

The possibility of extending hypovirulence to other pathogens

Biological control of other pathogens, using low virulence variants, is an attractive prospect for phytopathologists. The low virulence variant is poorly adapted, since the plant is able to reject it by using its own natural defenses. If it is to survive, therefore, it must possess some other selective advantage, that is, it must:

- either, have a selective advantage that will allow it to dominate normal forms of the parasite when competing with them;
- or be infectious, thereby transforming normal forms;
- or be able to create a state of immunity in the plant, so that, when it is inoculated before the arrival of normal forms, the plant is protected.

In the first case, control would take place at the level of the pathogen germ population; in the second case, during the process of infection; and in the third case, at the preventive level. We have been able to illustrate the second case by demonstrating the possibility of biological control of *Endothia parasitica*. This was done by using a contagious variant. Modification of the germ population of the parasite seems to be a relatively unexplored area, except in the field of microbial antagonism, and, even here, organisms other than variants of the parasite are used.

In our opinion, one prime advantage of using variants of the parasite belonging to the same species as the pathogen lies in the adaptation of these variants to the same ecological conditions experienced by the virulent form. Any ecological condition that favors the virulent will also favor the variant. In the case of *Endothia parasitica,* the variant is able to completely eliminate the disease, and is, to some extent, the ultimate weapon. In certain regions of Italy, *Endothia* has completely disappeared from chestnut plantations. We feel that this happened because two forms of the same species of parasite were involved.

In the area of biological control, then, the use of nonadaptive contagious variants would appear to be the most promising method. It remains to be seen how and in what conditions it can be used. In the first place, the contagious variants should, of necessity, lack one of the functions essential to the initial harmless stages of the infection. Either that, or the absence of such a function should delay infection long enough for the plant to react defensively, as in the case of *Endothia.* In the case of contagious hypovirulence, it should be easy for anastomosis to take place between the normal strain and the variant. This poses the problem of strain specificity, and again, we faced this problem when dealing with *Endothia* (functional anastomosis between different species of fungi is extremely unpredictable). However, we have found ways of overcoming vegetative incompatibility between strains and similar processes could be attempted when dealing with specific incompatibility.

In addition, the determinant of the variation should be epistatic in action, that is, "suppressive" in the case of cytoplasmic determinants. The spread of the character (physical diffusion and replicative reproduction) should take place very quickly. Finally, the variant should be able to disseminate itself by natural means. The advantage of extrachromosomal variants over nuclear variants is clear. Indeed, variants affected by a virus appear to be ideally suited to control of this kind. It remains to be seen how they can be obtained.

Three possibilities are open to us:

1. If virus-infected variants are involved, we could attempt to modify existing viruses in such a way as to make them infectious to other fungi;
2. The infectious mutants may exist in the germ population of the parasite. It would be possible to carry out a systematic search for these variants. We should then have to provide them with a selective advantage, probably by introducing them in the medium, so as to increase their inoculum potential;
3. The infectious mutants may be obtainable by mutagenesis. Since we are looking primarily for extrachromosomal mutants, we should have to use mutagens which act specifically on extranuclear determinants (acridines, tetrazolium chloride, erythromycin, etc.). May we say that this is not simply wishful thinking.

You will appreciate the necessity for scientific cooperation at the international level, if solutions to these problems are to be found.