FACTORS IN THE RESISTANCE OF CHESTNUT, CASTANEA Spp TO THE CHESTNUT BLIGHT, ENDOTHIA PARASITICA

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SUMMARY

Factors of disease resistance and disease endurance of chestnut Castanea spp. attacked by Endothia parasitica, the chestnut blight fungus, were studied. Water, alcohol and other extracts were made of different portions of the bark of Castanea dentata, the American chestnut, Castanea crenata the Japanese chestnut, and the Chinese chestnut Castanea mollissima. The extracts were made up in potato-dextrose agar plates and assayed with mycelium of the fungus. The water extract and the water soluble fraction of the alcohol extract had a retarding effect on the growth of the fungus. The extracts from the most resistant species, C. mollissima, was the most toxic, followed by the extracts of the somewhat less resistant C. crenata. The extracts of the highly susceptible C. dentata had little or no effect on the growth of the fungus. The alcohol soluble extractives and the ether extract also retarded hyphal growth, but the relative toxicity of these two extracts showed no correlation with the relative resistance of the three species.

The tannins of the bark of the three species were purified. They all retarded the growth of the fungus when present above a certain concentration. There was no difference between the effects of the tannin from Japanese and American chestnut, but that from the Chinese chestnut was much more toxic.

The total concentration of the tannins in the bark of three species is not correlated with the relative resistance of the species. In the water extract of the barks, however, the concentration of tannins was larger in extracts from the resistant species. It is suggested that this is an indication of a differential solubility of the tannins in the three species.

A qualitative test seems to indicate that the tannin in Chinese chestnut is a pyrogallol-tannin, whereas the other two species contain a mixture of catechol- and pyrogallol-tannin.

It is suggested that the relative resistance of the three species, at least in part, is the result of the differential solubility and qualitative differences between the tannins in the three species studied.

A study of the effect of different concentrations of a complete nutrient solution on the resistance of chestnut indicated that the nutritional level effects resistance in a complicated manner. In an early stage of infection, perhaps at the stage of the actual penetration, the plants are more resistant the higher the concentration of nutrients (within the limits studied); at later stages, however, resistance appears to decrease with increasing concentration of nutrients. Explanations for this phenomenon are suggested.

TABLE OF CONTENTS

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INTRODUCTION

In the breeding for disease resistance in plant crops the problem immediately arises: How is the relative resistance of the new strains to be determined? It has been pointed out by Newton et al. (1929) that the plant breeder is handicapped when attempting to solve this problem and has to rely largely on empirical methods because of our lack of knowledge regarding the nature of resistance to plant diseases.

In the chestnut breeding work which was started in Hamden, Connecticut some twenty years ago, the difficulty involved in determining the relative resistance of the new hybrids has been a serious obstacle.

Young chestnut trees which are growing under natural conditions often escape infection by the chestnut blight fungus Endothia parasitica. This can be observed in the forests in Connecticut. In stands which formerly contained a large proportion of chestnuts one can now find many suppressed but uninfected trees as much as 30 years old. When, however, these trees are finally infected by the fungus they show no resistance to the disease and die within a year or two, as evidenced by the many similar trees which are dead or dying in the stands.

In the plantation in Hamden, experience has shown that the trees usually live to an age of 8 to 12 years before they show any signs of infection and until then it is impossible to obtain information regarding their resistance and their value in the future breeding program.

During the first years of the breeding work artificial inoculation was tried to overcome the problem. The technique was difficult and the results too unreliable. Trees, which repeatedly had been inoculated without positive results, would later become infected naturally and would prove to have little or no resistance. Other workers have had similar results, thus W. C. Bramble, has informed the writer that he often found it impossible to successfully inoculate young native trees, which later showed no resistance to the disease. It was, however, not a case of immunity of all young seedlings.

Dr. A. H. Graves, who has been promoting the chestnut breeding at the Sleeping Giant Plantation in Hamden, later tried to establish a correlation between the total sap concentration of the bark and the resistance to the blight fungus. No such correlation was apparent. The seasonal and diurnal variations in the individual tree were found to be greater than the variation between resistant and non-resistant trees.

When the present writer started to work on the chestnut breeding program at the Connecticut Agricultural Experiment Station, in cooperation with Dr. A. H. Graves, it was decided that to obtain the greatest efficiency in the breeding program it would be necessary to find a method by which the resistance of the trees could be determined at an early age.

To do this it would be necessary to understand the environmental factors influencing resistance and the inherited

characters which cause some trees to be resistant. When more was known on these points it was thought that some technique could be developed by which the true relative resistance of a tree could be determined in the seedling stage.

After a study of the different types of disease resistance in plants and the factors which may effect the host-parasite relationship it was decided to approach the problem from two different points of view.

First of all it seemed possible that some chemical compound present in the bark of the trees may be the cause of the resistance. This could probably be shown by extracting the bark and assaying the extracts with the blight fungus.

Secondly, it seemed possible that the plants would show varying degrees of susceptibility when grown under different nutritional levels. Perhaps a level could be found at which a clear picture of the relative resistance of the trees could be obtained in the seedling stage.

This thesis is a presentation of the results of the study as outlined above.

The Chestnut Blight Fungus *Endothia parasitica* (Murr) Anders (Ascomycetes sphaeriaceae)

History

The chestnut blight fungus was first found in 1904 in New York City (Anderson, 1914), but from there it spread rapidly over the entire area in eastern America where the American chestnut *Castanea dentata* has its distribution.

Today most of the old native chestnuts in this area are either dead or are attacked by the fungus.

In the first years after the discovery of the fungus there was much discussion regarding its origin. One group of scientists led by Clinton of the Connecticut Agricultural Experiment Station claimed that it was a native fungus (Clinton, 1913), which had attained its serious parasitic character only because the host trees had been weakened by adverse environmental conditions e.g., severe winters, prolonged droughts and by continued coppicing for a long period. The other point of view, which was held by Metcalf (1908) and Anderson and Rankin (1914), emphasized that the disease was spreading from areas in which Japanese chestnuts had been introduced as early as 1876. They felt that the rapid spread of the disease from certain centers of infection was an indication that it was caused by an introduced fungus to which the native chestnut showed no resistance. They were finally proven to be right, when the fungus was found in China and Japan and identified as E. parasitica by Shear and Stevens (1913 and 1916).

Hosts

Endothia attacks all species of the genus Castanea, but the susceptibility of the different species varies considerably. The American chestnut is very susceptible, and the European chestnut, Castanea sativa, is only slightly less susceptible. Of the two oriental species the Chinese

chestnut, C. mollissima, is the most resistant; only rarely will the fungus kill a tree. Cankers will usually heal over before they reach any considerable size. Occasionally nurseries list selected immune trees for sale; so far, however, the writer has never seen any Chinese tree that did not show some slight sign of the disease eventually. Castanea crenata, the Japanese chestnut, is also resistant to the disease, but not to the same degree as the Chinese chestnut. Frequently Japanese chestnut trees are so badly cankered that they have to be cut down.

The fungus is found as a parasite only on chestnuts and chinquapins, but it has been found as a saprophyte on other native hardwoods such as oaks, shagbark hickory and staghorn sumac (Boyce, 1938, Anderson and Rankin, 1914). Anderson and Babcock (1913) inoculated many native species of trees and found some parasitic development on staghorn sumac, white oak and chestnut oak. They conclude, however, that "attack on the other species is of importance only in that they may be the means of keeping the fungus over in a locality where the diseased chestnut has all been destroyed".

Dissemination and Infection

How the fungus was spread from tree to tree and from one locality to another was the subject of much discussion in the early days of the fight against the rapidly spreading disease. The results of extensive experimentation were summarized by Anderson and Rankin (1913) and unless otherwise

stated the following discussion is based on their report.

Man was an important agent in spreading the disease from locality to locality, and spot infections found far ahead of the main advance of the fungus could often be traced to nursery stock which had been brought in from areas which the fungus had already reached. The fungus may also be spread from diseased to healthy trees on the tools of the cutting crews. No exact information has been obtained regarding the role played by logs, poles, posts, etc., which were transported to new areas, but it is quite likely that they were of some importance, as the fungus can remain alive as a saprophyte on such material for a considerable length of time.

There can be little doubt that birds aided in the spread of the disease, and the same undoubtedly was true in the case of insects, although the most important role of the insects is that they cause wounds through which the infection may develop.

Rain is one of the agents that spread the pycnospores. These spores are sticky and develop in "spore-horns"; they become quite brittle when dry, and therefore are resistant to the wind. The rain dissolves the "spore-horns", and carries the spores to spots lower down on the stem or to other trees nearby.

Experiments indicate that wind-carried ascospores are the most important cause of the rapid spread of the fungus. The ascospores are ejected from the perithecia to a distance

of as much as 22 mm. (Anderson and Babcock, 1913) and are easily picked up and carried by the wind. Ejection only takes place when the perithecia are thoroughly wet, but they remain active for several hours after a heavy rain.

Infection can take place only through wounds that are deeper than the outer green cortex. Any type of wound is sufficient. Anderson and Babcock (1913) in a few cases succeeded in causing an infection after a period of 10 weeks on sound bark, but the condition of the experiment made an unusually favorable environment for the fungus and they conclude that "all other experiments indicate that the cases where the fungus enters through sound bark are so rare as to be entirely negligible".

Pycnospores will germinate within 18 - 36 hours at temperatures ranging from 60-75°F.; with higher temperatures the germination is quicker, and 89°F. was found to be the optimum. This may indicate that the hot periods during the summer are most favorable for infection by pycnospores.

Ascospores germinate in from 6 - 12 hours at room temperature, but germination of 25 per cent was obtained at temperatures as low as 38°F. within twenty four hours.

It is important to note these time limits, as they may be an explanation why young trees escape the disease. The young smooth bark furnishes less favorable conditions for germination than old bark because it dries out so rapidly that the spores are unable to germinate. If the young trees

have been entered by the fungus they are, however, as susceptible as the older trees.

The Development of the Canker on C. dentata

When the fungus has gained entrance to the bark it appears to thrive on the dead and injured cells until a mass of mycelium has been produced. It then starts to penetrate the surrounding bark with what appears to be purely mechanical force. The mycelium forms a fanlike mass which forces the cells apart. It is not clear whether any enzymes or toxins produced by the fungus take part in the process.

W. E. Keefer (1914) made a study of the pathological histology of the fungus and he formed the opinion that the advance was a mechanical rather than an enzymatic action. In no instance did he observe the penetration of the cell walls by individual hyphae. Bramble (1936) however considers two steps in the invasion of the bark. The primary invasion is accomplished by the mass action of the mycelium fan, whereas the secondary invasion is a penetration of individual hyphae into the cells on the sides of the fan. This penetration is "accompanied by certain microchemical changes in the infected and affected cells". A lignification of the cell walls take place and the tannins in the cells are oxidized by oxidases secreted by the fungus. It appears from the paper that some enzymes are secreted into the tissue ahead of the advancing mycelium, but Bramble does not apparently consider them a factor in the penetration itself. This

would then indicate that their action is limited to the hydrolises of the plant materials to digestible foods.

Not all the bark tissue is destroyed by the fungus. From Keefer's study it appears that the cork tissue both in the primary cortex and the "internal cork layers" is little affected by the fungus. The individual cells show no change, but they are forced apart by the force of the advancing mycelium fan. The sclerenchyma, consisting of bast fibers and stone cells, and the crystal-containing cells also remain unchanged. The crystal-containing cells are small cubical cells containing calcium oxalate crystals; the cells are arranged in regular rows.

The collenchyma lying just beneath the cork tissue is not disarranged by the fungus, but Keefer found that whereas at least partial lignification takes place in the walls of the cells a little back of the advancing edge of the fungus, no change could be demonstrated at the exact edge of the canker. Keefer looked for lignification in pathological tissue caused by other fungi, but was unable to find any and he concluded that the process is peculiar to the blight fungus. Bramble (1936) however, points out that other plants respond in the same fashion to wounding and he showed that lignification occurs in chestnut bark which has been wounded mechanically.

The parenchymatous tissue, the sieve tubes and the cambium cells all show partial or complete lignification,

according to Keefer; the cell contents show a decrease in the amount of starch and proteins and a considerable increase in tannin materials. These cells are all destroyed by the fungus and their lumens can be seen filled with masses of hyphae.

The fungus seems to stimulate division of the medullary ray cells and great masses of new cells are formed which may completely disarrange the surrounding tissue. The cells contain starch, proteins and small amounts of tannin; these compounds show no changes, and the individual cells are not affected by the fungus. Keefer did not observe the mycelium in the wood but was able to isolate the fungus from the sapwood at a depth of from 1 to 7 growth rings.

Bramble (1938) found that the hyphae penetrate to the innermost layers of the sapwood soon after the fungus has reached the cambial layer. The hyphae penetrate the sapwood through the wood and pith rays; later the ray parenchyma is invaded. No hyphae were found in the vessels until a very late stage when the tree crown is already partly dead. The conduction through the wood is not stopped by the direct action of the fungus, but the fungus seems to stimulate the parenchyma adjacent to the vessels to the formation of tyloses, which are the actual cause of stoppage of the flow.

Growth Rate of the Fungus

The growth rate of the fungus in the bark of American chestnut has been studied by Anderson and Rankin (1914) and

and Rankin (1914). The growth varies with the temperature, being most rapid in June, July and August when the average horizontal growth around the tree is slightly over 2 centimeters for a four week period; it may in some cases reach as much as 3 centimeters. Stevens (1917) states that the mycelium has no resting period; whenever the temperature rises above 8° or 9° C. it will resume growth regardless of the previous temperature. This agrees with Anderson and Rankin's data which give an average growth of 0.51 cm. for the month of January.

On artificial media the growth is much more rapid; the above authors mention an increase in diameter of 3 mm. per day, but as much as 12 mm. per day has often been recorded by the writer. The growth is also more rapid in the dying bark of a cut tree and it may be mentioned that no mycelial fan develops in dead bark.

Superficial Indications of Resistance

On young sprouts of the native chestnut which show no resistance the fungus will grow rapidly beneath the smooth outer bark. The bark is not broken and may be raised only slightly. The spreading mycelium can be seen as an orange discoloration under the thin bark.

When older sprouts are attacked the outer bark may seem to remain intact, because it usually takes so long for the fungus to kill the sprout that only when the red perithecia can be seen pushing through the destroyed bark can the fungus be identified.

The development of hypertrophied cankers and cankers with sunken centers and raised edges are definite signs of the host's reaction against the parasite.

On the least resistant trees the sunken cankers will be surrounded by an irregular line of callus mixed with dead tissue. The newly formed callus is continuously reinfected by the blight, which keeps on extending the canker. This is the type commonly found on the more resistant individuals of the native species and in most cases it is likely to be a result of favorable growing conditions rather than inheritable characteristics. Anderson and Rankin (1914) state that it is the type found on rapidly growing limbs. Until more exact information has been obtained, it is, however, the only criterion that can be used in selecting the parent trees in a breeding program.

A similar type of canker is found on Chinese and Japanese chestnut, but here the ridge of callus shows more resistance against reinfection and the wound will decrease in size rather than increase and will eventually disappear.

In the hypertrophied canker type the fungus is prevented from penetrating towards the cambium by the formation of wound tissue. This type of canker is common on all the three major species considered here. Wound tissue will check the advancing fungus only temporarily in the American species, but often walls off the fungus completely in Chinese and Japanese chestnut.

The Inheritance of Blight Resistance

Our knowledge regarding the inheritance of blight resistance is rather limited at present for the following reasons: (1) Although a large number of hybrids have been produced, the number of individuals representing any one cross is relatively small. (2) The parents of a species hybrid are heterozygous and a segregation takes place in the F_1 generation. This could be overcome by pure-line breeding. (3) In view of the fact that the chestnuts are self-sterile pure lines can be obtained only from repeated sibbings, a much slower process than selfing.

From our present knowledge we can say that disease resistance probably is the result of a multiple-factor inheritance rather than a one-gene difference (Graves, 1950). The pronounced variation in resistance within the wild species seems to indicate this. This variation could perhaps be explained on the basis of a number of mutant alleles determining resistance, but it seems doubtful that this is the case. It would result in a limited number of disease resistance classes in a hybrid, while actually our breeding records seem to indicate that the hybrids show a continuous gradation of resistance between the two parents. It is as yet impossible to arrive at an estimate of the number of factors involved.

There seems to be some indication that resistance is inherited after a somewhat different pattern in hybrids between the native species and the Japanese chestnut on the

one hand and between the native species and the Chinese chestnut on the other. It appears to the writer that, while in the Japanese-American hybrid the majority of the hybrid individuals approaches the latter parent in resistance the opposite is the case in the Chinese-American hybrid. The foundation for such a statement is weak indeed, but it seems that it is a question which should be studied in the future, as it may be an indication that more factors are involved in the disease resistance of the Chinese than in the Japanese chestnut.

We do not know what type of characters controlled by the genes are involved in the mechanism of disease resistance. Let us suppose that disease resistance is due to the presence of some chemical compound in varying amounts. Several different genes may very well control the formation of such a chemical and its formation leaves plenty of scope for explaining variation in resistance, either by the presence or absence of suppressor genes or by the formation over alternate pathways of diverse efficiency and controlled by different genes.

This would be a rather simple form of resistance, and it may well be that rather we should be thinking in terms of a type of resistance caused by a variety of characters controlled by individual genes and possibly their mutant alleles.

It has been pointed out by Graves (1950) that there is some evidence that disease resistance is linked with other

characters. In the Chinese-American and Japanese-American hybrid it seems that the resistance of the Asiatic species is closely linked to their inferior growth habit. This would be a serious obstacle to the development of a resistant timber tree because an F_1 hybrid comparable to the Asiatic parent in resistance in most cases will be valueless as a timber tree.

Some hybrids have, however, been developed which combine resistance with a good timber form. At present the most promising hybrid in the Sleeping Giant Plantation is the Chinese x (JapanesexAmerican) cross. More than 100 trees of this combination are now under observation and a large proportion combines a high degree of resistance with straight and rapid growth. It is difficult to obtain information concerning the inheritance of a character such as disease resistance from a complex hybrid like this. In the future, therefore, it seems that the breeding program should proceed along two main lines: (1) the further production and improvement of C x (JxA), backcrossing to selected native trees and intercrossing of selected individuals of C x (JxA). (2) Production of Chinese-American and Japanese-American hybrids on a large scale to study the inheritance of blight resistance if possible after some inbreeding of the parents.

Disease Resistance and Disease Endurance

When a plant through some inherent quality in its physical or chemical makeup is capable of more or less

successfully resisting the attack of a given parasite, this is defined as true disease resistance (Walker, 1924).

This definition excludes cases where plants for some reason escape disease, and those cases where the plant, because of some environmental condition favorable to its growth or unfavorable to the growth of the fungus, is able to endure the disease for some length of time.

The progress in our understanding of the host-parasite relationship has been slow in the past and many of the earlier conclusions have later been found to be wrong, because they were based on the evidence from experiments which yielded information regarding the effects of only one or a few factors on the relationship, whereas the actual responses were the result of the total effect of a series of closely interrelated factors.

The following discussion is not an attempt to give a full review of our present knowledge regarding the host-parasite relationship. Only experiments which have a bearing on the existing problem or which contribute to a general understanding of it, are discussed.

Disease Endurance

The environmental factors, such as soil fertility, temperature, humidity, and light conditions, may have a marked effect on the ability of a plant to endure a certain disease.

An interesting example, showing how a fungus attacking

two different host plants may react differently to a certain set of environmental factors, has been discussed by Dickson et al. (1923) in the case of the seedling blight Gibberella saubinetii. This fungus attacks both corn and wheat. It grows well over a wide range of temperatures from 3° to 28° C. Wheat develops best at soil temperatures of from 8° to 16° C., while corn grows best at 24° - 28° C. The wheat plant is most resistant at the relatively low temperatures which are optimum for its growth; the situation is reversed in the case of the corn, it shows most resistance at the relatively higher temperatures. Resistance in this case is also correlated with soil moisture; at 30 per cent of the moisture holding capacity of the soil both species are blighted regardless of temperature.

Further study indicated a close connection between the composition of the host tissue and disease development. At low temperatures the wheat seedlings have a high content of sugars and dextrins and show lower values at the higher temperatures, whereas in corn the correlation is reversed. The more carbohydrates available in the plants, the more resistant the plants will be, because the cell walls will be thicker and hence, more difficult for the fungus to penetrate.

A relationship between the temperature and nutrition and its influence on the resistance of the garden pea to Fusarium wilt has been described by Schraeder and Walker (1942). They grew the plants at different concentrations of a complete nutrient solution and found that at 21° C. disease

development decreased with increase in nutrient concentration. At 27° C., however, the highest degree of resistance was encountered at "normal" nutrient concentration; weaker, as well as stronger solutions, led to increased disease development, with the most severe damage at the highest concentration of the nutrient solution.

Cook (1937), Walker and Foster (1946) and Stoddard and Dimond (1948) studied the influence of different nutritional levels on the infectivity and virulence of the Fusarium wilt of tomato. Their results show that there is an optimum nutritional level for the infection and the development of the disease on the plant; this lies in the region between the minimum for normal growth of the plants and the ideal. The effect of a change in the balance of the various nutrient elements was also studied by Cook and by Walker and Foster. Cook found a high frequency of infection in plants where nitrate was applied and a low frequency of disease symptoms where it was omitted. No wilting of the resistant variety occurred under either set of conditions. This agrees with the results of Walker and Foster. In addition the latter authors found that a low application of potassium resulted in increased development of disease.

Walker and Kendrick (1948) studied the bacterial canker of tomato. A comparison of this studied with the study of the Fusarium wilt shows that the response of a disease to the nutritional conditions of the host plant may be specific. The bacterial canker of tomato is a phloem disease and reacts

to the nutrient level in a way opposite to that of the Fusarium wilt, that is, the disease symptoms increase with increasing nutrient concentration.

Similar differences were found by Walker and Hooker (1945 a and b) in their work with cabbage yellows and cabbage clubroot. Here the development of the disease increases with an increase in the nutritional level in the case of cabbage clubroot, whereas the opposite is true in the case of the cabbage yellows. Changes in the potassium and nitrogen levels also affected the development of the disease. Thus, omission of potassium caused a pronounced drop in disease development; this had been pointed out earlier by Pryor (1940).

The difference in the reaction of different diseases on the same plant to the nutritional condition of the plant has been explained by Walker and Kendrick (1948) on the basis of a competition between the host and the parasite for some particular essential element, or combination of elements, present in limited amounts. The reaction of the parasite will depend on, (1) its location in the host, (2) the nutritive requirements of the parasite and, (3) the availability of inorganic nutrients in the xylem in contrast to the plant metabolites in the phloem. The Fusarium wilt is a xylem invader and it thrives well on a medium where nitrogen is available as nitrate. When enough nitrate is available for both plant and fungus the disease symptoms will be limited, but if the nitrate supply is

limited at the low nutrient levels the fungus will "out compete" the host and the latter will show severe injury.

Disease Resistance

When discussing true resistance to disease it is necessary to distinguish between, (1) mechanical resistance and, (2) physiological resistance. Mechanical or structural resistance comprises the cases where a group of plants show resistance because of some peculiarity in their anatomy which prevents normal development of the parasite.

Willaman et al. (1925) thus found that plums resistant to brown rot have a tougher skin and denser flesh than susceptible varieties. Both factors decrease rapidly in effectiveness as ripening progresses, but the decrease is most pronounced in susceptible varieties.

Waxy coatings may be a factor in preventing the penetration of the hyphae into the host, and thickness of cuticle, size and number of stomata and development of cork layers may have a bearing on the degree to which the plant enjoys natural protection (Walker, 1924).

The formation of layers of cork around an area of infected tissue in plants is a common observation, as, for example, in apple scab and in the corky-scab disease of potato. How effective this cork formation is in preventing further spread of the fungus has, however, been subject to much discussion. It has often been found that a cork barrier which had formed in front of the advancing hyphae

later has been penetrated by the fungus, apparently constituting no obstacle to its advance (Thomas, 1934). Brown (1936) in reviewing the subject states that there is "some doubt as to whether cork barriers really function at all or merely mark the limit of spread of the parasite which has already been stopped by some chemical factor".

In several cases attempts have been made to establish a correlation between the physical conditions of the host and resistance. Thus, Newton et al. (1929) determined the following properties for eight varieties of wheat, some resistant and some highly susceptible to the rust: total solids, bound water, osmotic pressure, electrical conductivity, and pH. They could not, however, establish any relationship between these factors and resistance. Earlier Walker (1924) had pointed out that parasites are able to grow in solutions of much higher osmotic pressures than are encountered in the cell sap, and that osmotic pressure, therefore, could hardly be a factor in resistance. This has more recently been emphasized by Thatcher (1942 and 1943).

Thatcher made a thorough study of the effect of obligate parasitism on permeability of the cell membranes in the host. Working with a variety of fungi and hosts he found that when a susceptible plant is invaded by a parasite, a marked increase in the permeability of the semipermeable membranes of the host cell results. If, however, a resistant host plant is invaded, a pronounced decrease in permeability results. Narcotics have strong modifying effects

on this relationship; thus, a chloroform vapor treatment of a wheat variety which is resistant to a certain strain of Puccinia graminis will cause an increase in permeability rather than a decrease, and the plant will be more susceptible to the disease than is normally the case.

Thatcher suggests that the higher osmotic pressure of the fungus enables it to obtain water from the host cells, while the increased permeability of cell membranes makes solutes available to the fungus. If this is the case, a factor which modifies the permeability of the host cell should also modify the susceptibility of the host; that this actually is the case has already been mentioned. A decrease in permeability can actually explain resistance in a host plant, as it also would mean a decrease in the amount of solutes available to the fungus, the parasite ultimately starving to death.

The "toxin-antitoxin" theory may also be explained by these findings. The stimulation from the parasite causes an active response on the part of the host expressed as a decrease in permeability, the final result again being the starvation of the fungus. How the actual change in permeability is brought about has not been discovered as yet; it may be a modification of the plasma membrane due, perhaps, to dehydration. That it actually is a question of some chemical present in the resistant varieties, which is mobilized upon attack by the fungus is indicated by the work by Newton et al. (1929). They found that detached wheat

leaves from susceptible plants showed lesser amount of injury when growing partly submerged in extracts from resistant plants. Similarly, when juices from resistant plants were injected into the leaves of growing susceptible plants susceptibility decreased.

Newton and Anderson (1929) interpreted these results as a direct toxic effect upon the host cell; when the fungus enters the host cell, phenolic compounds are set free, killing the host cell, and in turn the parasite dies by starvation. The results could be explained equally well by the hypothesis suggested by Thatcher.

The work by Newton and Anderson leads to the subject of chemical resistance. According to this theory resistance is due to the presence in the host tissue of compounds which are toxic to the invader. The toxin may either be present in the host before the fungus enters, it may be one or more breakdown products, or it may consist of other compounds formed as a result of fungal stimulation.

One of the first workers to isolate a chemical from plant tissue and successfully prove its antibiotic effects towards a pathogen was J. C. Walker. Working with onion smudge, Colletotrichum circinans, Walker (1923) found that the volatile oil in expressed juice from the white susceptible varieties, as well as from the resistant pigmented strains, prevents germination of the spores of the fungus. This indicates that the volatile oils can not be the cause of the difference in varietal susceptibility. A study of

the infection in resistant varieties indicated that the effective agent was located in the outer dry, pigmented scales of the onion, while the disease spread as rapidly in the white, succulent scales as in the scales of the non-resistant onion. When small pieces of the pigmented scales were added to drops of distilled water in which spores normally germinate freely, highly abnormal germination resulted; if added to drops with growing mycelium, further growth was prevented. This suggested that the toxin was water-soluble. It was further found that the compound was thermolabile, live steam for 20 minutes being sufficient to destroy its toxic effects.

With this information Walker and his co-workers started their attempts to isolate the toxin and were able, by a series of extractions and precipitations, to isolate protecatechuic acid as pure crystals (Link, Angell, and Walker, 1929). As the extractions were made with neutral solvents at room temperature, and chemical tests showed very little alteration during extraction, the protecatechuic acid apparently exists as such in the plant. The amount of acid obtained was, however, not enough to account for the toxic effect of the original water extract (Angell, Walker, and Link, 1930). After further investigation catechol was also isolated from the pigmented onion scales. Although the phenolic acid first isolated showed an inhibition concentration of 1:800 the catechol had an inhibitory effect in a concentration as low as 1:1600 (Link and Walker, 1933).

The phenolic compounds are commonly found throughout the plant kingdom and their role as antibiotic agents against fungi has often been suggested. Walker and Link (1935) determined the inhibiting concentration of a great many phenols and related compounds against Colletotrichum circinans and other onion parasites.

As their figures are of interest in the following discussion they have been listed below as they apply to C. circinans.

TABLE 1. MINIMUM CONCENTRATION OF SOME PHENOLIC COMPOUNDS REQUIRED TO INHIBIT THE GROWTH OF C. CIRCINANS

Phenol	1:1600	Hydroquinone	1:800
Catechol	1:1600	Phloroglucinol	1:100 or more
Resorcinol	1:400	Pyrogallol	1:12800

It will be seen that the toxicity of the compounds varies considerably. Furthermore, the study showed that the tolerance of different fungi towards phenols in general varies considerably, and that the relative tolerance of the fungi studied varied from one compound to another.

From this study it is clear that the mere presence of some free phenolic or related compound in a plant is not sufficient evidence that the compound is the cause of resistance against some particular parasite. The nature of the compound must be determined, and a bioassay undertaken

with the fungus in question.

A similar more recent study has been conducted in Sweden by Erdtman and Rennerfelt. They studied the phenolic compounds of the heartwood of Scotch pine (Pinus sylvestris) and obtained two compounds, pinosylvin and pinosylvin monomethyl ether in pure form. Both compounds have a fungicidal effect on wood-decaying fungi such as Polyporus annosus. Pinosylvin seems to have a more general fungicidal effect, whereas the monomethyl ether is more specific, affecting only some of the decay fungi. Pinosylvin is effective in concentrations from 0.02 to 0.002 per cent (Rennerfelt, 1945). The compounds are found in most of the hard pines (Diploxylon) in a varying ratio, the ratio in Scotch pine being between 1:3 and 1:4. The relative resistance to decay appears to be correlated with this ratio. In the soft pines (Haploxylon), which have been investigated so far only the monomethyl ether of pinosylvin has been isolated (Erdtman, 1949).

Antibiotic substances, the so-called α, β and γ thujaplicins, have been isolated from western Redcedar, Thuja plicata, (Erdtman and Gripenberg, 1948; Rennerfelt, 1948). These compounds are not phenolic, but contain a seven-carbon-atom ring.

In several cases it has been discovered that plants owed their resistance to fungi or bacteria to alkaloidal compounds. Greathouse and Watkins (1938) isolated berberine from Mahonia trifoliata and M. Swaseyi and found that it

occurred in sufficient concentration in the plants to explain this resistance to the Phymatotrichum root rot. Another alkaloid, sanguinarine, was isolated from Sanguinaria canadensis and its antibiotic effect against Phymatotrichum demonstrated (Greathouse, 1939).

It has often been emphasized that whenever chemical resistance is studied, it is not enough only to isolate the toxin from the plant and prove its antibiotic effect against the fungus in question. The quantity in which it occurs in the plant and its localization in the host tissue must also be determined (Rigler and Greathouse, 1940).

Host-parasite Relationship in the Present Case

A valuation of our knowledge regarding the relationship between Castanea and E. parasitica from the standpoint of disease resistance and endurance

The Possible Causes of Disease Resistance in Chestnut

No comprehensive study of the cause of resistance of chestnut towards the Endothia canker has been made. The earliest study pertaining to the subject was carried out by Cook and Wilson (1915). They studied the effect of various tannin-containing extracts of the American chestnut on the growth and spore germination of E. parasitica. The study was an elaboration of earlier work by Cook and Taubenhaus (1911).

For the study an agar medium was used, to which glucose, peptone, potassium phosphate and magnesium sulphate had been added. The tannins were added in concentrations of

from 0.1 to 2.8 per cent. The results of the investigation are unfortunately somewhat confusing, as no exact data are presented. From the summary, however, it appears that, (1) 0.8 per cent tannin, regardless of its type, retards the germination and frequently causes an abnormal stimulation of the growth of the aerial mycelia, (2) E. parasitica was able to use as much as 2 per cent tannin as food, once it had been established on the medium. Two specially prepared pure tannins were used; one consisted of the water-soluble tannin fraction of the bark, whereas the other fraction was soluble in alcohol as well as in water. The first fraction had a stimulating effect on the parasite while the second had a tendency to retard it. A third extract comprising the coloring matter of the bark "was extremely toxic" to E. parasitica. On a medium combining the water-soluble tannins and the coloring matter, the toxicity of the latter was, however, largely overcome. A fourth extract was employed. It contained fractions of the above mentioned extracts (60 per cent) plus 10 per cent fermentable sugar, 7 per cent gallic acid, 8 per cent pentoses and pentosans, 5 per cent water plus 10 per cent undetermined. The extract represents about 9 per cent of the dry weight of the bark. This was the most toxic extract assayed. Growth was slow the first week; in 10 days growth was good on cultures containing up to 2 per cent extract. From then on growth was very slow and the mycelium was discolored to an ashen gray (Cook and Wilson, 1916). No information is given in the

paper regarding the procedure which was used in obtaining the various extracts or to any chemical tests to which they might have been subjected. It is therefore impossible to draw any conclusion about their chemical nature.

The roots of American chestnut are resistant to the canker fungus (Graves, 1926). A comparison of inoculations of roots and shoots of comparable thickness showed that growth of the fungus was very much retarded in the living root while it grew rapidly in the shoot. Graves suggests as a possible explanation the very high tannin content of the roots. Samples, which he had analyzed, showed a tannin content double that of the shoot.

Bramble (1936) from his study of the bark of C. dentata, which was invaded by the fungus, came to the conclusion that resistance may be due to the formation of cork layers bordering the diseased tissue. However, since he observed these layers penetrated by the fungus in some cases, he suggested, that in order to be checked in its advance by the cork layers the fungus must first be weakened by some compound present in the host cell.

Keefer (1914) found that the ray parenchyma cells in diseased bark developed into great masses of undifferentiated tissue and suggested that they may play a role in checking the advance of the fungus.

All the above studies have been limited to C. dentata and in none of the cases was there an attempt to distinguish between disease resistance and disease endurance. Conclu-

sions with regard to the possible causes of resistance were drawn from results of inoculations on ordinary seedlings of the American chestnut. No selections of especially resistant trees were made. It is therefore possible that what were considered indications of resistance were normal reactions for all trees, the extent to which they develop being an expression of disease endurance. The studies are, however, interesting in that they may give us an idea of where, or where not, to look for the mechanism of true disease resistance as it appears in the Asiatic chestnuts. As such they seem to bring forward the possibility of a resistance due to either mechanical or physiological causes or perhaps a combination of both.

The Possible Role of Tannins in Blight Resistance

It may at this point be of interest to consider briefly the chemical composition of the bark of the most important species of chestnut. It has been difficult for the writer to gather information on this subject as it has appeared largely in publications of the leather industry which were not available to him.

Kurth (1947) mentions the components of the bark of C. sativa as follows: Ceryl alcohol, fatty acids, phytosterol with a melting point of 133-135° C., resins, invert sugar, and tannins. No similar data have been found in respect to the other species, and they alone are hardly enough to permit an interpretation. They appear to indicate

that no alkaloids are found in the chestnuts, and that a possible chemical factor in resistance is more likely to be found among the phenolic derivatives or perhaps the fatty acids.

An examination of data on the concentrations of tannins in the different species indicates that resistance cannot be explained simply by the higher concentrations in the resistant individuals. Some of the available data have been summarized in the following table.

TABLE 2. DISTRIBUTION OF TANNINS IN THE BARK OF FOUR IMPORTANT SPECIES OF CHESTNUT

	Root		Stump or root crown		Lower Trunk	
	Range	Aver.	Range	Aver.	Range	Aver.
	Per cent Tannins (Dry Wt. Basis) in the Bark					
1. C. mollissima	26.1-30.2	28.4	17.0-24.8	20.6	13.3-17.8	16.0
2. C. crenata					10.9-20.1	14.4
3. C. dentata	25.4-37.7	31.4			10.3-13.6	12.7
4. C. sativa			up to 14			

1. Clarke and Frey (1932). 15 years old.
2. Unpublished data from J. D. Diller's sample. 49 years old.
3. Frey and Leinback (1925). Trees forty years, and older.
4. Wilson (1928). Data questionable.

The average values for the tannin content in the lower trunk of Chinese, Japanese, and American chestnut indicate

a correlation between the tannin content of the bark and the relative resistance of the three species. The ranges in the tannin content overlap considerably. This would indicate a similar overlapping in the relative resistance of the three species; a condition which is not found in nature. Therefore, resistance cannot be correlated to the total tannin content of the bark.

It may be objected that the data do not allow any interpretation at all, as they are compiled from analyses of material from trees of different ages. However, Clarke et al. (1942) indicate that no important changes take place in the concentration of the bark tannins during the life of the tree. Some effects may be expected from differences in environment; they should, however, be relatively small as all the trees in question grew in the vicinity of Washington, D. C.

That the concentration of the tannins can not directly account for the disease resistance does not mean that the tannins cannot be the cause of resistance. This has been pointed out by Offord (1940). He studied tannins in reference to Ribes and Cronartium ribicola and could not demonstrate any direct correlation between tannin concentration and resistance to the fungus. By empirical tests he did, however, find that the highly susceptible R. petiolare contains tannins belonging to the depsides or gallotannins (Perkin's classification), the moderately susceptible R. inerme contains tannins of this type as well

as catechol-tannins. This agrees with Kargaplova (1937) who found that wheat species immune against Puccinia triticina also contain catechol-tannins.

If such differences between susceptible and resistant strains could be proved to be of common occurrence it would offer plenty of scope for an explanation of disease resistance.

Disease Endurance in Chestnut

Only one experiment pertaining to the influence of inorganic nutrition of chestnut on disease endurance has come to the writer's attention. Diller et al. (1946) studied the effect of mineral nutrition on the susceptibility of 50-year-old Japanese chestnuts to the blight. Some of the trees were treated with two different amounts of nitrogen, some were treated with potassium and phosphorus, and some were treated with different combinations of the three chemicals. An application of 300 pounds nitrogen per acre, applied once, brought about a significant increase in shoot growth; further application caused no additional increase in growth. All other treatments were without effect on the rate of growth. With respect to the endurance of the canker fungus, it appears that an application of phosphorus and potassium one year prior to the inoculation made the trees more susceptible. The authors suggest that this may be the result of a toxic effect of the chloride ion (K being applied as KCl) on the growth of the trees. The application

of nitrogen in addition to P and K modified the effect in a complicated manner.

In view of the fact the two strains of the fungus which were used responded quite differently to some of the treatments, the authors avoided statement of any definite conclusions. The results do, however, indicate that the reaction of the trees to the disease can be modified by manipulating their mineral nutrition.

None of the above experiments seem to favor any one of the approaches to the problem outlined in the introduction. To determine the relative resistance of hybrids at an early age it is necessary to determine the fundamentals of the causes of disease resistance. If these causes can be determined it seems likely that specific tests could be designed which would determine the resistance of the hybrids. On the other hand, it may be that by growing the plants under standard environmental conditions which are optimum for infection, inoculation tests could be made which were sufficiently accurate for the purpose.

MATERIALS AND METHODS

Bioassays With the Fungus in Extracts of the Bark

General Description of the Technique

In order to determine whether antibiotic substances were present in the bark of chestnut trees it was decided to make a comparative study of the rate of germination of

pycnospores in extracts from the bark.

First, assays were attempted in depression slides, but this had to be abandoned as it proved to be impossible to focus the microscope on the very small spores [1.25x3.56 microns (Anderson, 1914)] through the depth of the liquid in the well on the slide.

Then the writer sought to germinate the spores in small droplets on an ordinary microscopic slide, which had been covered with a 25 per cent solution of cellulose nitrate in butyl acetate. This solution will, when dry, prevent drops of water from spreading over the surface of the slide. This technique also had to be abandoned, partly because of the small size of the spores which resulted in difficulties in accurate counting of germination percentages, and partly because the slow rate of germination of the spores under the available conditions caused so much contamination that the spores of the fungus could not be distinguished from foreign spores. Finally, therefore, it was decided to use the older method in which the relative growth of the hyphae on a solid medium containing the extract to be tried is taken as indicative of the fungicidal effect.

The technique which generally was used can be described as follows: The standard amount of potato-dextrose agar (39 gm. per liter) was mixed with the bark extracts, or the required control mixture. The suspension was heated on a water-bath until the agar was completely dissolved and was

then poured into test tubes in aliquots of ten cc. The tubes with contents were next sterilized in the autoclave at 15 pounds pressure for 20 minutes and while still hot the agar poured into petri dishes. The test tubes were protected from cooling until the plates were poured; it was possible to pour as many as 150 without reheating the medium.

The inoculations were made from cultures of the fungus growing on a potato-dextrose agar medium. Whenever possible the inoculum for a complete experiment was taken from a single culture. Sometimes, however, it was necessary to use two or three cultures as inoculum, in which case they were always transfers from the same original culture, in order to eliminate the possibility of variation in the inoculum.

Standard amounts of inoculum were obtained by using a 5 mm. corkborer for cutting the disks. The plates were stacked on shelves in the transfer room. This room was not heated, but its location is such that it has a fairly constant temperature of about 70 - 75° F. The plates were not placed in any special statistical design, but were shifted around after each measurement in order to avoid any border effects.

Isolates of the Fungus

Three isolates of the fungus were used, as follows:

1. Isolated from a group of sprouts of American chestnut

growing at the plantation in Hamden, Connecticut. Isolation made in September, 1948.

2. Isolated from sprouts of American chestnut growing close to the group mentioned above. Isolation made in March, 1949.

3. Isolated from a group of sprouts growing in Hamden, about 4 miles northwest of the plantation. Isolation made in July, 1950.

Preparation of the Bark

The trees from which bark was taken can be described as follows:

Castanea mollissima: a 19-year-old tree growing at the plantation in Hamden. The tree had been attacked by the blight but showed the type of recovery which is typical for the Chinese chestnut.

Castanea cronata: a 17-year-old tree growing at the plantation in Hamden. The tree had been damaged by heavy frost and subsequently badly attacked by the Endothia canker. It did, however, show resistance toward the disease.

Castanea dentata: four trees apparently seedlings 25-32 years old, growing in Norfolk, Connecticut. They were 2-3.5 inches in diameter and badly suppressed. None of the trees had been attacked by the fungus but as numerous similar trees in the surrounding woods showed no resistance, it seems safe to assume that they too were not resistant but had escaped the disease. It is not uncommon to find trees

of this type in the woods in Connecticut.

All the material was collected in the fall of 1948. From part of this material the bark was stripped in its entirety. The habit of growth of the fungus in Chinese and Japanese chestnuts seems to indicate that the factor of resistance is concentrated in the inner living bark; consequently it was decided to separate the outer dead portion and the inner living bark from part of the trees. To do this the outer dark colored part of the bark was first removed, then a thin layer of the inner bark was removed and discarded, and, finally, the inner bark was removed down to the wood. The bark was placed in paper bags near a radiator and allowed to dry. It was then ground in a Wiley mill with a 15-mesh screen.

The Extraction of the Bark

In order to obtain information regarding the solubility of the compounds which might be present, and thereby an indication as to the general nature of the chemical substances involved, it was decided to use distilled water, 95 per cent alcohol, and ether as solvents. The extractions were carried out as follows:

Distilled water: Thirty five grams (oven dry basis) of bark was weighed out and 210 cc. distilled water added. After heating for two hours on a steam bath the liquor, while still hot, was filtered through a Buchner funnel with two layers of #1 filter paper. Suction was used to facilitate

the filtration. 100 cc. of the extract was measured out and the medium prepared as described under the general description of the technique.

Alcohol (95 per cent): Thirty five grams (oven dry basis) of bark was soaked for two hours at room temperature in 210 cc. of 95 per cent alcohol. It was then filtered as described for the water extraction and the filtrate was poured through the bark twice while on filter. The alcohol was evaporated under reduced pressure at 40-44° C. The residue was then allowed to dry overnight at reduced pressure. It was taken up in 100 cc. distilled water, but did not dissolve completely. The water was filtered off, made up to the original alcohol volume by the addition of water and the plates prepared as already described.

The undissolved residue was dissolved on the filter in 50 cc. of absolute alcohol. A certain amount of this alcoholic extract was added to 100 cc. of water and the mixture was left on a steam bath until it evaporated down to the original volume of water. The plates were prepared with this solution.

Ether: The original extract was prepared in a manner similar to that described for alcohol. The solvent, however, was evaporated on a steam bath at a temperature which did not exceed 45° C. The residue was dried overnight in a desiccator, and then dissolved in 10 cc. of ether. The plates were prepared as follows:

10 cc. aliquots of potato-dextrose agar solution were

sterilized in the autoclave and while still hot 1 cc. of the ether extract was added; the test tube was shaken vigorously and the plate poured. One cubic centimeter of pure ether was added to the control plates. By this method the ether evaporates and if anything is left it does not prevent normal growth of the controls.

Collection of Data

The cultures were measured at 24-hour intervals. The dishes were marked with a right angle cross on the bottom, and two diameters were measured along this cross. Their mean was used for the further computations. The measurements were made with a transparent millimeter scale which was illuminated from below. The diameter was recorded to the nearest millimeter; in cases of doubt the nearest lower millimeter was favored.

The raw data were ranked according to the date of record using uniform ranking values. If, for example, positive measurements were recorded for 9 consecutive days the ranking value for the first day was -4 followed by -3, -2, -1, 0, 1, 2, 3, 4; for 8 days of recording it would be -7, -5, -3, -1, 1, 3, 5, 7. The products of the ranking value (x^1) and the measurements (y) were summed to give $S(x^1y)$. In the tables the data have been presented by this term.

The average increment or regression coefficient (r) was determined as $\frac{S(x^1y)}{S(x^{1^2})}$.

The further statistical treatment differed from one experiment to the next and will be described as the results are presented.

The Results of the Experiments with the Crude Bark Extracts

Water Extract

An experiment using water as a solvent was started on December 16, 1949. The plates were ready for inoculation on December 20. A transfer of isolate #2 from December 3 was used.

The results of this experiment are given in TABLE 3.

To test the 10 series for homogenity the values were plotted in sequence from the lowest to the highest, with the rankits for ordinal data from Table XX in Fisher and Yates (1938) as the ordinates. All the series except C_E (Chinese, entire bark) and C_I (Chinese, inner bark) plotted as straight and approximately parallel lines indicating that their variation is homogeneous. If in C_E the zero value for plate #4 and in C_I all the zero values and the low value for plate #6 were disregarded, both lines did, however, correspond sufficiently closely with the others. In the statistical treatment of the data it was therefore decided to substitute the zero value with the curve value (379.0) in the C_E series. In finding the error term for the analysis all the C_I values were disregarded and in finding the combined sum of squares for species, bark posi-

ABLE 3. THE GROWTH OF E. PARASITICA IN WATER EXTRACTS OF DIFFERENT PARTS OF THE BARK OF CHINESE, JAPANESE AND AMERICAN CHESTNUT

The values in the table represent the term $S(x^1y)$

	Chinese			Japanese			American			
Test #	Inner bark	Outer bark	Entire bark	Inner bark	Outer bark	Entire bark	Inner bark	Outer bark	Entire bark	Control
1	421.5	409.0	416.5	439.0	369.5	417.0	562.0	422.0	491.5	536.5
2	0.0	384.0	423.5	429.5	376.5	406.5	562.0	389.0	492.0	538.5
3	0.0	377.5	448.0 (379.0)*)	422.0	363.5	406.5	566.5	350.0	491.0	557.0
4	424.5	377.5	0.0	435.0	364.5	414.0	578.0	389.0	459.0	528.0
5	0.0	389.5	465.0	419.5	352.5	407.0	544.5	411.0	493.5	537.0
6	291.5	393.5	450.5	408.0	364.0	402.5	529.5	409.0	475.0	515.5
7	0.0	400.0	406.0	401.5	333.0	408.5	563.0	405.0	473.0	546.5
8	405.5	412.5	416.5	424.5	359.0	419.0	556.5	405.0	475.5	533.5
9	0.0	403.0	455.0	433.0	341.5	418.0	570.5	403.5	491.0	527.0
10	388.0	419.5	391.5	424.0	366.5	415.0	571.5	385.0	495.0	536.0
(x^1y)	1931.0	3966.0	3872.5	4236.0	3590.5	4114.0	5604.0	3968.5	4836.5	5355.5
tted tals	3830.0		4251.5							
m)	3.218	6.610	6.454	7.060	5.984	6.857	9.340	6.614	8.061	8.925
ted r	6.383		7.086							

*) Plotted value

tions and their interaction, the total based on the plotted average was used.

The basic analysis of variance has been shown in TABLE 4.

TABLE 4. BASIC ANALYSES OF VARIANCE FOR THE BIOASSAYS OF THE CRUDE WATER EXTRACTS

Source of Variation	Degrees of Freedom	Sum Squares	Mean Squares	F Value
Species	3)			
	)			
	)	6720.82	746.76	7.445** x)
Bark position	2)			
	)			
Species x bark position	4)			
Error	80	8024.56	100.31	

x) ** indicates significance at the 1 per cent level.

The further analysis was made according to a factorial design and has been shown in TABLE 5.

The tests show clearly the difference between the native and the Asiatic species. In other words, there is strong evidence that the bark of the Asiatic species contains a water-soluble compound which has antibiotic properties.

Although the analysis does not indicate a difference

between the outer bark from Chinese and Japanese chestnuts, there is certainly a difference between the inner barks of the two species. The C_I extract completely prevented growth in 5 of the ten plates, while the J_I extract gave a uniform growth with r=7.060 mm.

The general effect of the extracts was a retardation of growth; A_I, however, did not affect the increment and may even have had a slightly stimulating effect.

The effect of the inner versus the outer bark is difficult to interpret, because the two portions of the bark behave differently in the different species. This is emphasized by the highly significant interaction (1 x 4).

The effect of the extract from the entire bark is intermediate between that of the extracts derived from the inner and the outer bark. This is what could be expected as it is a mixture of the two other portions of the bark.

The experiment with water extracts was repeated twice, but only with extracts of the outer bark and the entire bark. Isolate #2 was used for both experiments.

The results of these two experiments were not analyzed statistically, but the average increments or regression coefficients are shown in TABLE 6.

TABLE 6. AVERAGE INCREMENT (IN MM.) OF E. PARASITICA GROWN IN WATER EXTRACTS OF CHESTNUT BARK

	Chinese		Japanese		American		Control
Exp.#	Entire bark	Outer bark	Entire bark	Outer bark	Entire bark	Outer bark	pure p-d agar
1	0.0	5.743	1.310	5.959	7.856	7.012	8.585
2	2.379	6.894	6.568	8.324	8.224	7.878	9.128

The relative effect of the extracts is the same in both of these experiments and the first experiment indicates that the Chinese and Japanese chestnut barks undoubtedly contain materials extractable in water and possessing antibiotic properties. The effect of the different portions of the bark is not the same as in the first experiment, and it is, therefore, impossible to determine if the antibiotic materials are concentrated in a particular portion of the bark.

Alcohol Extracts

The extraction with alcohol was carried out at the end of January 1950. The experiment involved only extracts of the entire bark, as the samples of outer and inner bark were small.

The assay of the water-soluble fraction of the alcohol extract was started January 30, using a three-week-old

transfer of isolate #2 as the inoculum. The alcohol-soluble fraction was assayed beginning February 10, using a two-week-old inoculum of isolate #2. The results of the two assays are shown in TABLE 7.

These two tests do not lend themselves advantageously to a statistical analysis as a factorial, so the different series were compared by means of a standard "t" test. There was no significant difference between the two controls, the value being far below significance t= .919(d.f.14); the two experiments can therefore be compared directly.

The comparisons are summarized below:

Water-soluble fraction vs. alcohol-soluble fraction

Chinese vs.Chinese	Japanese vs.Japanese[1]	American vs.American
2.120	8.839**	5.768**

Water-soluble fraction

Chinese vs.Japanese	Chinese vs.American	Japanese vs.American
6.156**	16.399**	10.159**

Alcohol-soluble fraction

Chinese vs.Japanese	Chinese vs.American	Japanese vs.American
5.110**	2.851*	0.419

It is clear that all the extracts had a retarding effect on the growth of the fungus. The difference in growth between the control and the extracts was of such a magnitude that a statistical test was unnecessary.

1) ** significant at the 1 per cent level.
* significant at the 5 per cent level.

TABLE 7. THE GROWTH OF *E. PARASITICA* IN THE WATER-SOLUBLE AND ALCOHOL-SOLUBLE FRACTION OF ALCOHOL EXTRACTS OF THE BARK OF CHESTNUTS

	Water-soluble fraction				Alcohol-soluble fraction			
Plate #	Chinese	Japanese	American	Control for water frac.	Chinese	Japanese	American	Control for alc. fraction
	S(xly)	S(xly)	S(xly)	S(xly)	S(xly)	S(xly)	S(xly)	S(xly)
1	352.5	372.0	419.5	594.5	383.5	282.5	361.0	543.0
2	342.5	371.5	412.5	562.0	375.5	321.5	319.0	601.5
3	346.5	363.5	406.5	576.5	336.0	315.0	323.5	598.5
4	348.0	381.0	404.0	605.5	382.5	316.0	263.0	583.5
5	354.0	360.0	405.0	618.5	395.5	273.0	340.0	549.0
6	350.0	370.5	397.5	594.5	321.5	323.0	333.5	560.5
7	329.5	367.5	413.5	568.0	402.0	308.0	227.5	585.5
8	352.0	382.5	416.0	549.5	356.5	318.5	349.5	563.5
Total	2775.0	2968.5	3274.5	4669.0	2953.0	2457.5	2517.0	4585.0
r (mm)	5.781	6.184	6.822	9.727	6.152	5.120	5.244	9.552
SE	$\sqrt{7.873}$	$\sqrt{7.558}$	$\sqrt{6.616}$	$\sqrt{68.899}$	$\sqrt{102.275}$	$\sqrt{44.651}$	$\sqrt{262.873}$	$\sqrt{61.632}$
Average	346.88	371.06	409.31	583.63	369.13	307.19	314.63	573.13

From a comparison of the species it appears that the alcohol-soluble fraction has the strongest effect although the difference between the two fractions obtained from the bark of the Chinese chestnut is not significant.

The relative toxicity of the water-soluble fractions of the bark of the three species is the same as for the crude water extract (TABLE 3); their toxicity corresponds to the relative resistance of the species. The relative toxicity of the alcohol-soluble fractions did not follow this pattern.

Ether Extract

The ether extracts were assayed beginning January 30. A 3-week-old transfer of isolate #2 was used as inoculum. The results are shown in TABLE 8.

TABLE 8. THE GROWTH OF E. PARASITICA IN ETHER EXTRACTS OF THE BARK OF CHESTNUTS

Plate #	Chinese $S(x^1y)$	Japanese $S(x^1y)$	American $S(x^1y)$	Control $S(x^1y)$
1	277.0	7.5	0.0	572.5
2	248.0	0.0	37.0	541.5
3	281.0	0.0	94.5	547.5
4	0.0	0.0	332.5	619.0
5	0.0	0.0	312.0	536.0
6	0.0	0.0	0.0	544.5
7	0.0	0.0	358.0	554.5
8	0.0	2.0	197.5	622.0
9	2.0	2.0	203.0	606.0
10	88.5	189.5	171.0	556.5
Total	896.5	201.0	1705.5	5700.0
r (mm)	1.492	.335	2.843	9.500
SE	$\sqrt{1607.32}$	$\sqrt{354.83}$	$\sqrt{1832.74}$	$\sqrt{110.06}$
Average	89.65	20.10	170.55	570.0

It is apparent that the ether extracts of the three barks also contain some compound with antibiotic properties. The results of the "t" tests have been shown below, but in view of the extreme variations of the samples they must be used with caution in interpreting the results.

Ether extracts, "t" tests:

Chinese vs. Japanese	Chinese vs. American	Japanese vs. American[1]
1.570	1.379	3.217**

The effect of the extract of the bark of the Chinese chestnut occupies an intermediate position between the two other extracts, and the effect of the extract of the bark of the Japanese chestnut is significantly lower than that of the American chestnut.

All three extracts have strong antibiotic effects but as the magnitude of their effects does not follow the pattern of the natural resistance of the species, the extract probably does not contain any factors which play a role in the disease resistance.

A condition like this is not uncommon. Thus, Walker (1923) found that the volatile oil from the onion retarded the germination of the spores of the onion smudge fungus, but as the oils of both resistant and susceptible onions were equally effective he concluded that the oils were not the agent he was looking for.

From these three experiments it appears that there is an antibiotic factor or factors present in the bark of Chinese and Japanese chestnut, and that it is soluble in alcohol and more so in water.

Water is so universal in its dissolving characteristics, that it hardly eliminates any type of organic compounds as

1) ** significant at the 1 per cent level.

the possible agent. However, it seems that the fats, fatty acids, hydrocarbons and waxes which are dissolved by ether and not by water can be disregarded (Kurth, 1947). Further, since the factor is found in the fraction of the alcohol extract which is soluble in water this eliminates at least the phlobaphenes and resins, as these compounds are precipitated from alcohol solutions by water, i.e., they are insoluble in water (Procter, 1908).

According to Kurth (1947) the water extract will predominately contain tannins, starch, pectins, sugars and glucosides. Of these it seems most likely that the toxic agent will be found among the tannins, the glucosides, or related compounds, but other compounds may very well be present which could account for the effects of the extract.

In order to fractionate the extract further the following schedule was set up:

1. Determine the moisture content of the bark.

2. Extract 100 gm. of bark with 1000 ml. 95 per cent alcohol, by soaking at room temperature for 24 hours. Condense to small volume at reduced pressure and add 100 ml. cold water to precipitate resins, gums and phlobaphenes.

3. Dry and weigh resins, etc., and assay with fungus.

4. Assay water extract.

5. Shake water extract with dry ether in a separatory funnel. This will remove gallic acid if it is present and probably other impurities. Assay ether fraction and the remaining water fraction.

6. Precipitate water fraction with lead acetate removing tannins, phenols and possibly alkaloids and some impurities. Assay the filtrate.

7. Dissolve lead precipitate with H_2SO_4 avoiding an excess. Filter off $PbSO_4$ and assay the filtrate.

8. To one half of the above filtrate add common salt to saturation and shake with ethyl acetate in separatory funnel. Filter and remove ethyl acetate at reduced pressure. By this method a fairly pure tannin should be obtained in the form of a porous mass.

9. To the remaining half of the water fraction add twice its volume which is a mixture of two-thirds 10 per cent acetic acid and one-third 5 per cent lead acetate. This should precipitate the pyrogallol-tannins more or less completely, while the catechol-tannins remain in solution. Dissolve the lead precipitate as under item 7 and assay.

This schedule, however, soon proved to be impractical because the ether (item 5) formed a fine emulsion which separated extremely slowly. Furthermore, the ether formed addition compounds presumably with the tannins in the extract. These compounds are very insoluble and as they upset the schedule it was given up.

Considering the high tannin content of the bark and the controversial literature on the role of tannins in disease resistance, it was decided to aim directly for the purification of the tannins and an assay of these compounds with the fungus. Determination of whether or not tannins were the

source of resistance to disease would be of general value in the study of disease resistance, and, positive or negative, the results would be a step toward the goal in this particular problem.

The Purification of Tannins

A considerable amount of work has been done on the purification of tannins, but only little progress has resulted due to the complex nature of their chemistry and their non-crystalline characteristics. It was finally decided to use the technique which was developed by Kurmeir (1927) and recommended by Freudenberg (1932) for the purification of the tannins of chestnut and oak. The technique was modified slightly in order to use the available equipment with most efficiency.

One thousand grams of the entire bark was soaked 2 hours in 3500 ml. distilled water on a steambath; the infusion was stirred repeatedly. It was then pressed in a hydraulic press. Two to three liters distilled water heated to 60° C. were added and the bark was again pressed. The addition of water and the pressing was repeated three times. After this treatment the bark gave a very weak reaction for tannins with iron alum.

The extract was filtered through paper pulp and the filter carefully washed with water. The tannins were precipitated from the extract thus obtained with a concentrated lead acetate solution; excess lead acetate was

avoided. The precipitate was filtered off in a Buchner funnel, washed with 4 liters of water and dissolved with the required amount of 10 per cent sulphuric acid. The resulting precipitate of lead sulphate was removed on a pulp filter and carefully washed. The process of precipitation with lead acetate and subsequent washing was repeated three times.

Barium hydroxide (0.1 N.) was added to the final solution until the excess sulphuric acid was removed. The barium sulphate, which was formed, was left in the solution because it facilitates the precipitation with pyridin.

This solution was precipitated by adding 400 cc. of pyridin; the precipitate was rapidly filtered, and dissolved with 12 per cent acetic acid and again filtered. A small amount of dark colored, high-molecular-weight precipitate remained undissolved; it was discarded. The acetic acid filtrate was precipitated with lead acetate and dissolved in sulphuric acid as before. The solution is again precipitated with pyridin and the procedure just described repeated. Finally the two filtrates from the pyridin precipitations and the solution of lead salts dissolved in acetic acid are combined and the mixture precipitated with lead acetate; by precipitating the tannins from this pyridin-containing solution a considerable amount of impurities remain in solution. They are removed by careful filtration and washing of the precipitate. It is then dissolved in 10 per cent sulphuric acid (no excess) and once again precipitated

with pyridin. A small precipitate is formed, and removed, and the remaining solution precipitated with lead acetate and dissolved in 10 per cent H_2SO_4.

This tannin solution was concentrated to about 500 cc. and, after thorough chilling, precipitated with a suspension of quinoline (22 ml. quinoline in 478 cc. distilled water). Excess quinoline should be avoided as it may lead to a "gummy" precipitate which is hard to filter. The suspension was filtered rapidly and the precipitate dissolved in 15 per cent acetic acid. This tannin solution containing acetic acid was immediately precipitated with lead acetate and regenerated. The filtrate from the quinoline precipitation contains a small amount of tannin and some impurities; the filtrate is precipitated with lead acetate, regenerated and again chilled and precipitated with quinoline. This quinoline precipitate is dissolved with 15 per cent acetic acid, again precipitated with lead acetate, regenerated and finally added to the main solution.

To remove the remaining small amount of impurities the solution is concentrated to 250 ml. and extracted for a total of 72 hours with deacidified ethyl acetate. The extraction was carried out in a continuous flow apparatus similar to the one shown by Freudenberg, but it was not possible to use a vacuum as the water was not sufficiently cold to prevent the ethyl acetate from escaping. Instead, the temperature of the ethyl acetate was raised to 80° C., at which temperature the apparatus worked well.

The extracts of the three different species react quite similarly to this purification schedule, the only exception being the precipitation with pyridin. While the extract from the Japanese chestnut formed a large flocculent precipitate which readily settles out, the extract from the American species forms a precipitate which consists partly of large flocculent particles which settle, and partly of very small particles, difficult to remove from the liquid. In the case of the extract from the Chinese chestnut the entire precipitate consisted of small particles similar to those described for the American chestnut bark extract.

The final purified tannins were superficially alike, being dark brown, amorphous compounds.

No attempt was made to keep the procedure quantitative. The final yield of tannin was small, between 2 and 2.25 grams for the Chinese and Japanese species and slightly under 2 grams for the American (only 700 g. of bark of this species were extracted initially).

Result of the Assay of the Purified Tannins

The pure tannins were assayed in four concentrations, starting with 1.2 per cent and diluting three times to get each subsequent concentration. Expressed as percentages the concentrations are approximately 1.20, 0.40, 0.13 and 0.04. The media were made up as described for the crude water extract; the required amount of tannins dissolved in water is mixed with the potato-dextrose agar and heated until the agar has dissolved, the medium is finally auto-

claved and the plates poured.

Nine plates of each concentration were prepared. The plates were poured December 5, 1950 and inoculated the next day with a two-week-old transfer of isolate #3 as the inoculum. The results of the assay are presented in TABLE 9.

The measurements were started December 8, 1950 and terminated on December 14. No growth was recorded for the 1.2 per cent tannins of Chinese chestnut during this interval, although all cultures showed signs of weak growth activity, too irregular to measure. On December 18 these cultures were measured again and showed an average diameter of 10.33 mm. The growth of the fungus on the 0.4 per cent medium of tannin from Chinese chestnut was quite abnormal; instead of spreading out radially from the inoculum long narrow arms spread very rapidly from the center, and from them the intervening spaces were gradually filled. This can be seen in Figure 1. The measurements from this series have been left out as they were incomplete.

This abnormal growth on the 0.4 per cent medium and the retardation of growth on the 1.2 per cent medium was not of a permanent nature. Transfers were made from these plates on December 18 to standard potato-dextrose agar media; the resulting cultures were normal in all respects.

The data presented in TABLE 9 show that the tannins derived from the bark of Chinese chestnut were much more toxic than the extracts from the bark of Japanese and American chestnut. The two latter extracts retarded the

TABLE 9. THE GROWTH OF E. PARASITICA IN DIFFERENT CONCENTRATIONS OF PURIFIED TANNINS DERIVED FROM THE BARK OF CHINESE, JAPANESE AND AMERICAN CHESTNUT

	Chinese				Japanese				American				
Test #	.04%	.13%	.4%	1.2%	.04%	.13%	.4%	1.2%	.04%	.13%	.4%	1.2%	Control
	S(xly)	S(xly)		S(xly)	S(xly)	S(xly)	S(xly)	S(xly)	S(xly)	S(xly)	S(xly)	S(xly)	S(xly)
1	279.5	276.0	..	0.0	314.5	305.0	282.5	227.5	298.0	289.5	289.5	248.5	287.5
2	289.5	249.5	..	0.0	300.0	310.0	283.5	249.0	298.5	282.0	281.5	248.5	289.0
3	278.5	291.5	..	0.0	301.0	302.0	280.0	234.5	298.5	287.0	298.5	244.0	276.5
4	280.5	279.0	..	0.0	287.0	302.0	277.0	238.0	308.5	281.0	283.5	242.5	296.5
5	285.0	304.0	..	0.0	289.0	306.0	276.0	245.0	309.0	294.0	288.0	239.0	285.5
6	290.0	276.0	..	0.0	287.0	304.5	280.5	243.0	322.0	295.5	281.5	253.0	284.0
7	284.5	285.0	..	0.0	308.0	291.0	284.0	223.0	305.0	300.0	296.5	237.5	283.0
8	293.0	281.0	..	0.0	300.5	300.0	278.0	239.5	307.0	304.0	291.0	252.5	293.0
9	287.0	289.0	..	0.0	301.0	277.0	275.5	255.0	308.5	288.5	283.0	250.0	286.5
Total	2567.5	2531.0	..	0.0	2688.0	2697.5	2517.0	2154.5	2755.0	2621.5	2593.0	2215.5	2581.5
r(mm)	10.188	10.045	..	0.0	10.667	10.704	9.988	8.550	10.933	10.403	10.290	8.792	10.244
SE	$\sqrt{2.854}$	$\sqrt{24.500}$	..	$\sqrt{0.0}$	$\sqrt{9.992}$	$\sqrt{11.091}$	$\sqrt{1.152}$	$\sqrt{11.338}$	$\sqrt{6.331}$	$\sqrt{6.722}$	$\sqrt{4.492}$	$\sqrt{3.569}$	$\sqrt{3.722}$
Average	285.28	281.22	..	0.0	298.67	299.72	279.67	239.39	306.11	291.28	288.11	246.17	286.83

growth of the fungus only in 1.2 per cent media, whereas, the tannins from Chinese chestnut, as mentioned, prevented growth temporarily at the 1.2 per cent concentration and resulted in abnormal growth at a concentration of 0.4 per cent.

No differences were apparent between the effects of the extracts from the Japanese and American chestnut.

The effect of concentration and the effect of the different extracts were also apparent from the pycnospore formation on the different cultures as discussed below.

The cultures were examined January 8, 1951 when they were slightly more than a month old. The following description is based on this examination:

Control: Good development of spore pustules. The pustules are not arranged in concentric rings, but in irregular, tongued formations around the center.

The 0.04 and .13 per cent media of all species: Abundant formation of spore pustules; there are more than on the control, and they have a tendency to aggregate in concentric rings.

The 0.4 per cent media: All species show fewer spore pustules than the two weaker solutions, and perhaps slightly less than the controls. There is some aggregation in rings. The number or pustules is nearly the same in the extracts from American and Japanese

chestnut, or possibly somewhat smaller for the extract from the latter species. On the tannins from the bark of Chinese chestnut, there are definitely fewer than on the control and the other extracts.

The 1.2 per cent media: Relatively few pustules are scattered over the cultures. Here again there appear to be slightly fewer on the extracts of the Japanese chestnut.

Representative cultures on the 0.4 and 1.2 per cent media are shown in Figure 1.

The Concentration of Tannin in the Crude Water-Extracts

In view of the evidence, that the purified tannins, when present in certain concentrations, have a retarding effect on fungal growth, it appears to be of interest to determine the tannin concentration of the crude water extract. For this purpose extracts of the inner and the outer bark of the three species were prepared according to the technique which has been described. Cultures of the fungus were made from part of the extract. Six plates were prepared for each extract.

The remaining extract was analysed for its tannin content according to the standard hide-powder method, which is used by the leather industry (A.O.A.C., 1945). The writer is indebted to the Department of Analytical Chemistry of the Connecticut Agricultural Experiment Station for this part of the work.

FIGURE 1

Representative cultures of E. parasitica growing on the purified tannins from the bark of American, Japanese and Chinese chestnut.

A. 0.4 per cent tannin.

1. American chestnut

2. Japanese chestnut

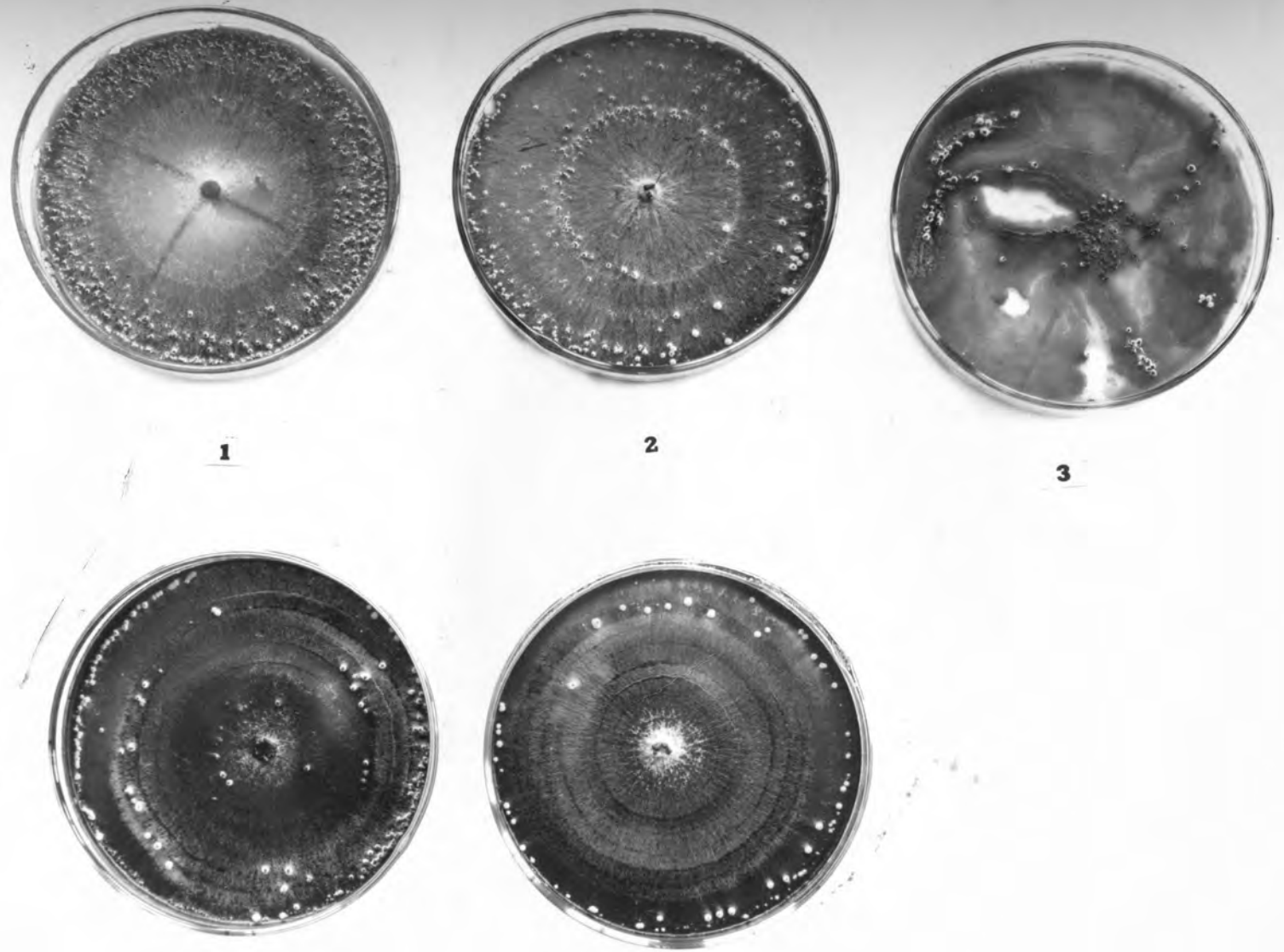
3. Chinese chestnut

B. 1.2 per cent tannin.

1. American chestnut

2. Japanese chestnut

The white spots with dark centers are the spore pustules. Note the abnormal growth on the medium containing 0.4 per cent tannin from Chinese chestnut.


A
1
2
3
B

The test was made twice, with the only difference that a 31-day-old transfer was used as the inoculum in test #1, while it was 15 days old in test #2. Isolate #3 was used.

The tannin content of the extracts in the two tests is given in TABLE 10.

TABLE 10. TANNIN CONCENTRATION OF THE CRUDE WATER-EXTRACTS OF THE BARK OF THREE SPECIES OF CHESTNUT

Species	Per cent Tannin in Extracts			
	Inner bark		Outer bark	
	Test 1	Test 2	Test 1	Test 2
Chinese chestnut	1.32	1.30	1.39	1.51
Japanese chestnut	1.01	1.07	.52	.59
American chestnut	.60	.74	.55	.60

The corresponding average increment expressed as the regression coefficient (r) is shown in TABLE 11.

TABLE 11. THE AVERAGE INCREMENT, IN MILLIMETERS, OF E. PARASITICA GROWING ON WATER EXTRACTS OF CHESTNUT BARK

Species	Average Increment			
	Inner bark		Outer bark	
	Test 1	Test 2	Test 1	Test 2
Chinese chestnut	1.550	10.491	5.179	8.286
Japanese chestnut	3.064	10.360	4.718	8.232
American chestnut	6.886	11.226	8.619	9.387
Control	11.188	11.226	11.188	11.226

The percentages of tannins in the two experiments agree quite closely. Apparently the tannins in outer bark of Chinese chestnut are somewhat more concentrated, or more soluble, than in the inner bark, while for Japanese and American species it is the inner bark that produced the higher tannin concentration. The values for the outer bark of Japanese chestnut are not in accordance with the values for the two other species, and are difficult to explain. It may be that for some reason tannins have leached out of the bark, but to explain the difference between the three species it will be necessary to assume that due to some factor, perhaps a peculiarity in the bark structure, the leaching occurs only in the bark of Japanese chestnut.

The values for growth increment differ considerably from test #1 to test #2. This difference is undoubtedly due to the difference in the age of the inoculum. In other experiments a dependence between the age of the inoculum and the reaction to the extracts has been observed, the effect of extracts increasing with increasing age of the inoculum.

Although not conclusive, the tests seem to indicate a correlation between the concentration of the tannins in the medium and the growth of the fungus. This is especially apparent from the extracts of the inner bark, and is supported by the results from the assays, already presented, of the other water-extracts in which the tannin content undoubtedly was comparable to the values given in TABLE 10.

For the outer bark this correlation is not apparent, as the average growth of the fungus on the extract derived from Japanese chestnut is much the same as on that obtained from the Chinese species while the tannin concentration is similar to that of the extract from American chestnut. This may possibly be explained by the presence in the bark of Japanese chestnut of some other compound different from tannin and toxic to the growth of the fungus.

It is, however, the inner bark which is most interesting to us because from field observations this seems to be the part of the bark which is able to prevent further advance of the fungus.

Qualitative Tests of the Extracts

The crude bark extracts and the purified tannins were subjected to a set of tests, which are used by the leather industry as means of identifying different vegetable tannins. The tests are of an empirical nature, but help in classifying the tannins in a number of broad classes. The tests which were used here were those described by Atkin and Thompson (1937) and by Freudenberg (1932).

The tests and the reactions which were obtained have been summarized below.

The Iron Alum Test: 3 to 5 drops of a 1 per cent iron alum are added to 2 to 3 cc. of the tannin solution of analytic strength (0.4 per cent tannin).

Crude extract: All extracts give bluish-black

coloration. Perhaps the color is somewhat greenish black in case of the extract from American chestnut.

Purified tannins: The color is hard to determine, it appears to be bluish-black although it may be somewhat more green with the extracts from the bark of American and Japanese chestnut.

The Bromine Test: Bromine water (4-5 gm. bromine per litre) is added drop by drop to 5 cc. of a 0.4 per cent tannin solution.

Crude extract: No immediate precipitate in Chinese or American chestnut bark extract. The extract from Japanese chestnut forms a light yellow precipitate.

Purified tannins:

No precipitate with any of the extracts.

The Acetic Acid-Lead Acetate Test: Add to 5 cc. of the 0.4 per cent tannin solution, 10 cc. of 10 per cent acetic acid, and 5 cc. of a 10 per cent lead acetate solution.

Crude extracts: All extracts give immediate precipitation. The filtrates give blue precipitates with iron alum and the precipitate is larger in the extract from the American and Japanese species than in the extract from the Chinese species.

Purified tannins: All extracts give immediate precipitation and the filtrate gives a blue color with iron.

The Formaldehyde-Hydrochloric Acid Test: Add 10 cc. of 40 per cent formaldehyde and 5 cc. of concentrated hydrochloric acid to 50 cc. of tannin solution (0.4 per cent). The mixture is boiled for 1/2 hour under a reflux condenser. Cool thoroughly and filter. Add 10 cc. of the filtrate to 5 gm. of solid sodium acetate and 1 cc. of 1 per cent iron alum.

Crude extract: The extract from the Chinese chestnut yields a small precipitate after boiling. The filtrate gives a purple color with iron alum. The extract from the American and Japanese species both give immediate precipitates, before heat is applied. With iron alum they both yield purple colors as in the extract from the Chinese species.

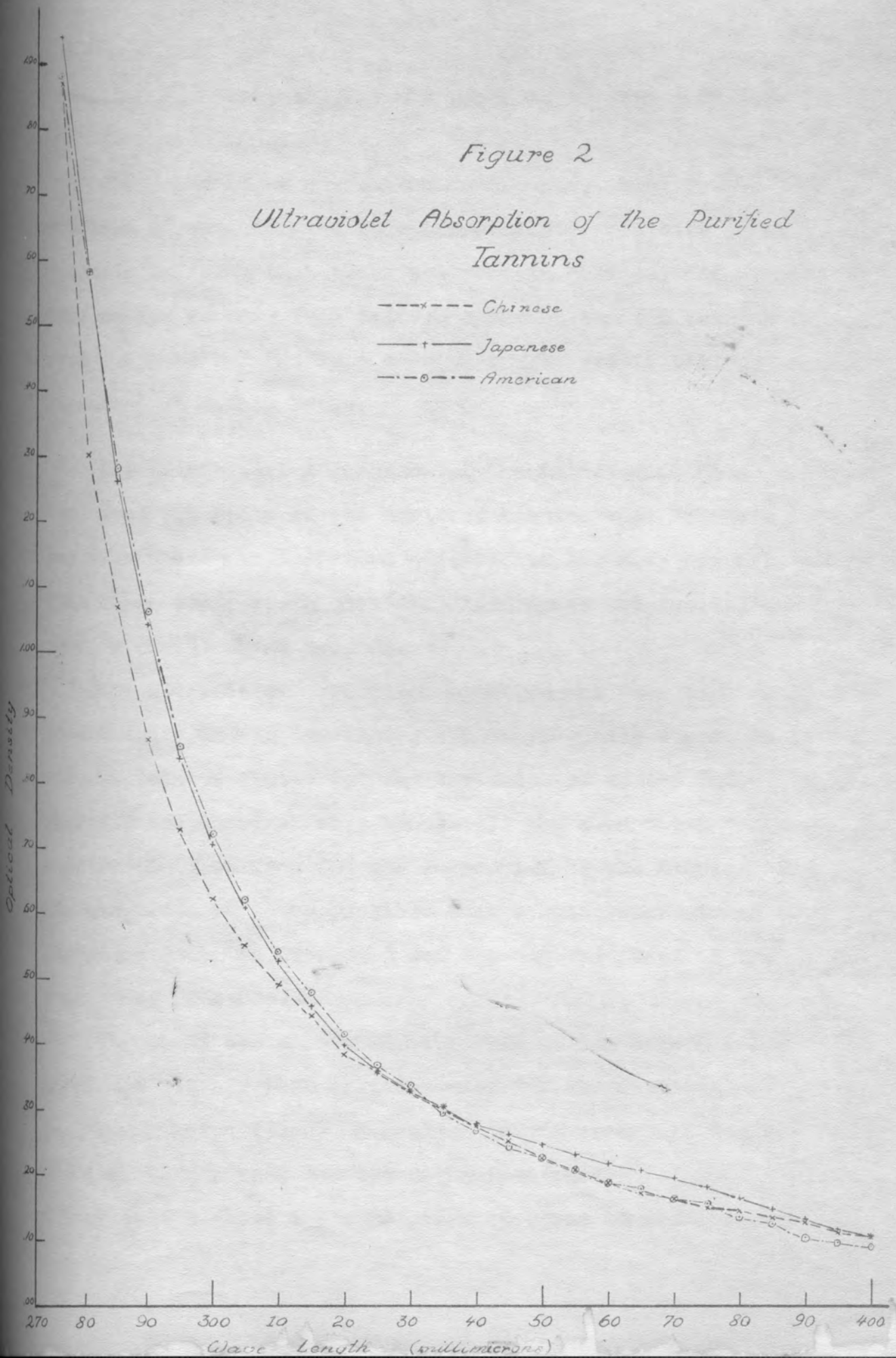
Purified tannins: The Chinese chestnut tannins yield no precipitate during boiling or upon subsequent cooling. With iron alum the filtrate yields a black or very dark violet color. The American and Japanese chestnut tannins do not form any precipitate upon boiling, but a heavy brown precipitate is formed when the liquid is cooled. With iron

FIGURE 2

The ultraviolet absorption of the purified tannins.

Figure 2

Ultraviolet Absorption of the Purified Tannins



alum they give a brown color with a violet tinge.

The point to be emphasized in the above test is the reaction of the "formaldehyde-hydrochloric acid test". It seems to indicate that there are certain chemical differences between the tannins from Chinese chestnut and the tannins from Japanese and American chestnut. The result will be discussed in detail later.

The Ultraviolet Absorption of the Purified Tannins

From the assay of the purified tannins with Endothia and from the above described qualitative tests it appears that there are certain chemical differences between the tannins of the three species.

The ultraviolet absorption spectrum has been used successfully in distinguishing the polyphenolic compounds in closely related fruits such as varieties of apples and cherries (Johnson, et al., 1950). If the absorption spectra could be used for the separation of the tannins of the chestnut, it seems possible that a relatively simple technique could be developed for the determination of the resistance of a tree.

To obtain the absorption spectrum of the tannin solution, the 0.4 per cent solution used for the qualitative test was diluted 1:100. A Beckman Quartz spectrophotometer Model D. U. was used for the determination.

Figure 2 shows the absorption spectrum of the three

tannins; in the graph the optical densities are plotted against the wavelength in millimicrons. The curves are very similar for all the three tannins, and show no distinct absorption peaks.

Toxicity of Different Phenolic Compounds

It has been suggested that the antibiotic effects of tannins were due not to the tannins as such, but to the phenolic compounds formed by the enzymatic activity of the fungus (Kargaplova, 1937; Offord, 1940; Newton et al., 1929).

It has already been mentioned that different species of fungi differ in their response to the same and to different phenols (Walker and Link, 1935). It therefore seems of interest in the present study to determine the effects of different phenols on E. parasitica.

Five of the common phenols were tried: pyrogallol, catechol, protocatechuic acid, gallic acid and phloroglucinol, and in addition commercial tannin or tannic acid (a digallic acid). Each compound was tried in 4 concentrations as follows: 1:400, 1:1600; 1:6400 and 1:25400.

The technique employed was a modification of the one which was used for the assay of the tannins. The required amounts of the phenols were dissolved in absolute alcohol to make up the concentrated solutions. Standard potato-dextrose agar in 10 ml. aliquots was autoclaved in test tubes. Immediately after the sterilization 0.25 ml. of the concen-

FIGURE 3

The effect of different concentrations of pyrogallol on the growth of *E. parasitica*.

1. Control; standard potato-dextrose agar.
2. Control; potato-dextrose agar to which 0.25 ml. absolute alcohol has been added per 10 ml. of medium.

3, 4, 5 and 6. Pyrogallol in the following concentrations: 1:25400, 1:6400, 1:1600 and 1:400.


1
2
3
4
5
6

trated solutions were added, the tubes were shaken vigorously and finally allowed to solidify in a slanting position. The tubes were prepared January 5, 1951 and inoculated on January 8 with a three-week-old transfer of isolate #3. The inoculum was similar to those used in the assay of the tannins; the agar disks were placed near the upper edge of the medium. Two controls were used in the experiment, one consisting of standard potato-dextrose agar and one to which 0.25 ml. of absolute alcohol had been added. The results of the experiment have been shown in TABLE 12.

It will be seen that the addition of 0.25 ml. absolute alcohol per 10 ml. of agar has a definite retarding effect on the growth. Thus, the control to which alcohol had been added covered 36.33 mm. on an average as compared to 51.50 mm. on the pure potato-dextrose agar.

Of the phenolic compounds pyrogallol was clearly the most toxic, showing complete inhibition in the two strongest solutions, and with a pronounced retarding effect on growth as well as on the formation of spore pustules in the two weaker concentrations. Figure 3 shows representative cultures of the different concentrations of pyrogallol compared with the controls.

In TABLE 12 an average growth of 0.50 mm. was recorded for the 1:1600 dilution on the tenth day of growth. Two cultures showed slight growth on this day, they did not develop any further and it therefore seems reasonable to

consider this concentration as completely inhibiting in its effect.

Next to pyrogallol, catechol was the most toxic compound. The 1:400 and 1:1600 dilution caused complete inhibition. The 1:6400 dilution was slightly retarding in the early part of growth, but later the growth rate was comparable to the control. This concentration did prevent the formation of spore pustules as shown in Figure 4. In the weakest concentration the compound had little or no toxic effect.

Phloroglucinol had some retarding effect in the 1:400 concentration and perhaps a little in the 1:1600. Growth in the two weaker concentrations appeared quite normal.

The three phenolic acids had apparently little or no toxic effect. The results with protocatechuic acid are not clear, as the 1:25400 dilution showed the strongest retarding effect (26.83 mm.) followed by the 1:1600 (27.50 mm.). The 1:400 (31.67 mm.) was intermediate between the above two concentrations and the control, and the 1:6400 dilution apparently had no effect. The response to digallic acid was very similar to that of the control and the gallic acid seemed to stimulate growth somewhat in the two strongest concentrations.

FIGURE 4

The effect of different concentrations of catechol on the growth of *E. parasitica*.

1. Control; standard potato-dextrose agar.
2. Control; potato-dextrose agar to which 0.25 ml. absolute alcohol has been added per 10 ml. of medium.
3, 4, 5 and 6: Catechol in the following concentrations: 1:25400, 1:6400; 1:1600 and 1:400.

DISCUSSION

From the results it is quite clear that the water extract and the alcohol extract of the bark of chestnut contain a compound, or compounds, which are effective in retarding the growth of E. parasitica when mixed into a standard potato-dextrose agar medium.

The relative effectiveness of the extracts follows the resistance of the three species as it appears in the trees in the field. Thus, the extract of Chinese chestnut bark is the most toxic to the growth of the fungus and that of the American chestnut the least, the extract of the Japanese chestnut being intermediate between the two in its effect.

The concentrations of the tannins in the crude extracts shown in TABLE 10, although inconclusive, seem to explain the effects upon the fungus, the toxicity increasing with increasing concentration of tannins. This, however, is not a complete portrayal of the relative resistance of the three species. It fails to account for the results of the assay with the purified tannins. Although this assay showed a correlation between the concentration of tannin and the toxicity of the medium, the relative toxicity of the tannin of the Chinese chestnut is so much greater than that of the tannins of the two other species that it becomes necessary to consider other factors in order to explain resistance completely.

The Solubility of Tannins as a Factor in Resistance

It has been pointed out that the total concentration of tannins in the three species of chestnut overlaps to such an extent that it cannot account for the relative resistance of the three species. However, the concentration of tannins in the extracts as shown in TABLE 10 may indicate that they have different solubility properties in the three species. It, therefore, is of importance to search the literature for information on the solubility of tannins and the conditions in which they occur in the plant cell.

The subject has long been studied by plant scientists in an attempt to discover why these compounds, which are noted for their precipitating effect on proteins, do not affect the protoplasm in the plant cell. The results have been summarized by Nierenstein (1934).

Lloyd (1911) studied the changes which the tannins in persimmon undergo as the fruit ripens and he suggested that the tannins in the idioblasts in the unripe fruit are present not in a true solution, but in a loose combination with a second colloid in the form of an emulsoid slime. In this condition the tannins are readily soluble and will be astringent to the taste. As the fruit ripens the two colloids combine more and more completely to form an insoluble gel. It is suggested that this change is due to the withdrawal of water. In the gel stage the astringent action of the tannins has been lost. Lloyd, according to Huber

(1929), found that the tannin in the bark of Quercus laurifolia is present in the form of an addition compound similar to what he found in the ripe persimmon.

Huber (1929) studied the solubility of the tannin in acorns and young oak seedlings. She found that a 40 per cent solution of acetone in water was the most effective solvent, while pure water was less effective, and absolute acetone, though a tannin solvent, failed to extract any tannin. This condition was undoubtedly due to the state of the tannins in the plant cell, and probably to an addition compound involving the tannin and some other compound as suggested by Lloyd. Such an addition compound could not be demonstrated microscopically in the acorn; they do, however, show clearly in the banana peel. This material reacts to extraction as does the oak material. This was considered good evidence that the tannin in oak was also present in the form of addition compounds.

It seemed possible that the behavior of the tannins in the dried dead material was different from that of these in fresh material, but Huber extracted sections of fresh material and got comparable results. She, therefore, considered her evidence conclusive.

Michel-Durand (according to Nierenstein, 1934) also made extraction studies of tannins in acorns using water and acetone as solvents. His results differ from Huber's in that he always found a fraction soluble in pure acetone, while the remainder would be soluble in water. He considered

the acetone fraction as "free" tannins occurring in solution in "Gerbstoffblasen", while the water fraction represented the "combined" tannins as described by Lloyd.

Nierenstein (1934) in discussing the work by Huber and Michel-Durand suggests that the difference in their results is due to differences in the preparation of material, and warns that it may be dangerous to draw conclusions from either investigation when this is considered. In his final statement he does, however, remark: "It is highly probable, even though Michel-Durand's conclusions may not be fully justified, that in many plants the tannin occurs partly free and partly bound or perhaps incompletely bound."

The solubility of the addition compound varies depending on how close the two colloids, which form it, are bound together. Huber, for example, found that the tannins in the banana peel were less soluble than those in her oak material. Nierenstein makes a statement to the same effect: "In the ripening fruit ..... (he refers to Lloyd's work with persimmon)..... the combination..... (between the two colloids)..... becomes more and more complete until an insoluble gel is produced."

The concentration of the tannins in the crude water-extracts (TABLE 10) is strong evidence that the tannins in the chestnut barks are present under similar conditions. It seems safe to assume, that had the tannins in the three species been equally soluble, their concentration would have been much more nearly the same. The situation

seems to be that the tannins in the American chestnut are present as an addition compound, bound closely together. In the Asiatic species "combined" tannins are loosely bound and therefore more soluble. It is also possible that the tannins are present partly in a "free" and partly in a "combined" stage, the proportion of "free" tannins being higher in the Asiatic chestnuts.

There is no doubt that the condition of the tannins in the cell would have a pronounced effect on the efficiency with which they would check the parasitic invader. The work by Huber (1929) is interesting in this connection. She first demonstrated that an agar - gelatin - or gum arabic-tannin complex behaves as the natural tannin addition compound when subjected to extractions as described above. She then proceeded to determine the rate of yeast fermentation of sugars, expressed as yield of carbondioxide, when a pure tannin solution and when a gum arabic-tannin complex was added to the sugar solution. It was found that although a pure tannin solution retards fermentation, it proceeds at a normal, or close to normal, rate when the gum arabic-tannin complex was added.

Other workers have reported similar results. Thus Cook and Wilson (1915) state that E. parasitica "gave good growth of mycelium and scant spore formation when grown on an agar medium containing 0.6 per cent tannin, but a less toleration to tannin when grown on a liquid medium". Bonnerfelt (1945) obtained the same results in his work with the

phenolic compounds isolated from pine.

Nierenstein (1934), discussing the work of Huber and Cook and Wilson concludes: "If the tannin in a host plant is present in the form of a compound with other colloids, it seems likely that it will have less inhibiting effect on parasitic fungi."

From the above discussion it will be seen that there is some evidence that the tannins, on account of their relative solubility, could be determining factors in resistance. This will explain the reaction of the tannins in the crude extracts of inner bark (see TABLE 10 and 11) but it does not explain the effects of the purified tannins. If one compares the concentrations of tannins in the crude extracts and the concentrations which were used for the purified tannins and the corresponding growth of the fungus it will be seen that the effect of the purified tannin of Chinese chestnut has about the same magnitude as the effect of the crude extract.

The pure tannins of Japanese and American chestnut, however, both seem to have a weaker effect than it would be expected from the crude extract. Although the tannin concentration of 0.60 per cent in the crude extract of American chestnut retarded the growth of the fungus considerably, as much as 1.2 per cent pure tannin retarded the growth only slightly.

The reason for this is not clear. The hidepowder method is known to be a rather empirical technique which

will include in the estimate of tannin related compounds, coloring matter, and the like. It will be remembered that Cook and Wilson (1915) found that while the tannin fraction soluble in water and alcohol retarded growth of the fungus somewhat, the coloring matter was "extremely toxic." It is quite possible that some of the impurities, perhaps coloring matter, in the crude extract which were estimated as tannin also possessed strong antibiotic effects; after their removal a higher concentration of the pure tannin would be required to bring about a comparable toxic effect.

The question still remains why the purified tannins of Chinese chestnut are so much more toxic than the other two. To answer this it will be necessary to look into the classification and some of the qualitative characteristics of the tannins.

The Classification of Tannins

The classification of tannins has been subject to much study; in the past many disconnected facts have been presented and different tests have been suggested. The result is that three different classifications are in use today:

1. Procter's classification considers two categories (Procter, 1903).
   A. Pyrogallol-tannins which give a blue color with iron salts, yield a "bloom" on leather and do not precipitate bromine.

B. Catechol-tannins, which give green colors with iron salts, contain a phloroglucinol nucleus, precipitate bromine and yield phlobaphenes.

2. Perkin considered three groups (Nierenstein, 1934)

A. The depsides which contain ester linkages. They give a blue color with iron salts.

B. Tannins derived from ellagic acid; they give green colors with iron salts.

C. Phlobaphene-yielding tannins. They give a green color with iron salts and contain the phloroglucinol nucleus.

3. A somewhat simpler classification and the one Nierenstein (1934) considers the most satisfactory is Freudenberg's (1932).

A. The hydrolyzable tannins which combine Perkin's group a and b. To this group belong the gallotannins, the depside tannins in which is found a chain of phenolic acids linked with ester linkages and finally the glucoside-like tannins. As the name suggests this group of tannins can be hydrolysed by acids and enzymes.

B. The condensed tannins, which are the same as Perkin's group C. These tannins can not be hydrolysed and they yield phlobaphenes. To this group belong the catechin tannins.

Freudenberg (1932) mentions that many natural tannins are incompletely understood and perhaps are examples of new classes. It is also possible that some natural tannins belong to both the hydrolyzable and condensed tannin group. In this connection it can be mentioned that it has been found more recently that certain fungi produce an enzyme system which can decompose the catechol or condensed tannins (Nierenstein, 1944).

Freudenberg (1932) also emphasizes that it is misleading to use the term pyrogallol-tannin as pyrogallol is also a component of the catechin tannins; also use of the term catechol-tannins for the catechin tannins is misleading.

The information regarding the nature of the tannins of the chestnut is limited. The tannins of the European species, C. sativa, have been studied more or less completely especially by the German investigators, but references regarding the three species considered here are very scanty.

From the studies of the European chestnut it is apparent that there is a pronounced difference between the tannins of the wood and the bark. This has been mentioned by Nierenstein (1934); and Atkin and Thompson (1937) state that whereas chestnut wood contains pyrogallol-tannins, the bark contains a mixture of pyrogallol- and catechol-tannins. Their statement refers to the American species. Similar statements have been made by Frey and Leinback (1925) and Greaves (1918) with regard to C. dentata. Ponte (1932 a

and b) studying C. sativa states that 25 per cent of the tannins in the bark are catechol-tannins whereas only 4 per cent are found in the carefully debarked wood.

Only one reference has been found regarding the type of tannin in the Asiatic species, and this only mentions the Chinese chestnut (Hsu, Chang and Tien, 1940). They consider the tannins of the leaves and bark of this species as belonging to the pyrogallol group. This publication was only available to the writer as an abstract, which gave no information regarding the tests which were used in classifying the compound.

The quantitative tests which were conducted in the present study seem to substantiate the classification which Hsu et al. (1940) give for the tannins of Chinese chestnut. These tannins gave a blue color with iron alum, and no precipitate with bromine; mixed with acetic acid they are precipitated with lead acetate and they give no precipitate with the formaldehyde-hydrochloric acid test. All these tests indicate that the tannin belongs to the pyrogallol-tannin group according to Procter's classification and, according to Freudenberg, to the hydrolyzable tannins similar to those in oak and chestnut wood.

The reactions of the tannin from the Japanese and American bark do not give a clear classification. This is undoubtedly due to a mixed nature. The formaldehyde-hydrochloric acid test indicates that the tannins from the Japanese and American chestnuts are mixtures. By this test

the catechol-tannins are precipitated while pyrogallol-tannins are only partially precipitated, if at all. If the latter tannins are present they can be demonstrated in the filtrate by the iron test. If both a precipitate and a positive color reaction are found it is evidence that the tannins are present in a mixture.

The acetic acid-lead acetate test is not conclusive but does not contradict the above mentioned results. Under the conditions of this test the pyrogallol-tannins are precipitated more or less completely while the catechol-tannins remain in solution and can be demonstrated by the green colors obtained with iron. The results obtained by the writer may very well be interpreted as indicating a mixture.

The iron test is not decisive. When mixtures are involved the color reaction is not reliable.

The result of the bromine test seems to be contradictory. The presence of a catechol tannin should, according to Atkin and Thompson (1937), result in a precipitate with bromine. This was not the case in this investigation, except for the crude extract of the Japanese bark. The many references which describe the tannins as mixtures do not mention the reaction to the bromine test. Frey and Leinback (1925) and Ponte (1932 b) apparently base their classification entirely on the test with formaldehyde-hydrochloric acid.

Freudenberg and Walpuski (1921), who studied the tannins

of C. sativa, reported that they did not obtain a precipitate with bromine. It should be mentioned that these authors, contrary to other investigators, consider the tannins of the leaves, bark, and stem of the same type.

To summarize the above discussion: Although the results are not entirely conclusive there seems to be some evidence that tannins of Chinese chestnut belong to the pyrogallol or hydrolyzable tannins, while the tannins of the Japanese and American chestnut bark are mixtures of pyrogallol-(hydrolyzable) and catechol-(condensed) tannins.

The Possible Correlation Between the Difference in the Tannins and the Relative Toxicity of the Purified Tannins

It has already been mentioned that the mere presence of tannins in a plant is not enough evidence to support the claim that they are factors in resistance. In addition their solubility, chemical nature, their distribution in the host plant, and the enzymatic activity of the fungus are involved.

Newton and Anderson (1929), among others, suggested that the toxicity of the tannins is caused by their phenolic constituents, which are released by enzymes secreted by the parasite. They were working with wheat rust, an obligate parasite, and were not thinking in terms of a direct toxic effect on the parasite. The liberated phenols kill the host cell, and thus the fungus is prevented from further growth.

Offord (1940), who also worked with an obligate parasite,

the white pine blister rust, contemplates a direct toxic effect upon the fungus. He considers the tannins absorbed in the cell walls the first barrier to the advance of the fungus. If the enzyme system of the fungus fails to convert these compounds into soluble or usable decomposition products, the haustoria die and further spread of the fungus is prevented. If, on the other hand, the haustoria do enter, Offord considers two possibilities. Either the tannins form a food source for the parasite, in which case growth may be stimulated, or the tannins are decomposed into toxic compounds by the action of the fungus, and the haustoria are killed.

It is important to note that the chestnut blight grows fungus well on dead tissue. This means that a destruction of the host cell by liberated phenolic compounds cannot effect the growth of the fungus, and chemical resistance must therefore be the result of a direct effect upon the parasite.

There is very little information in the literature concerning the relative toxicity of the different types of tannins. Offord (1940) and Kargapolova (1937) consider the catechol-tannins more toxic than the pyrogallol-tannins, because the pyrogallol-tannins are easily decomposed to phenols and phenolic acid, while the catechol-tannins are more stable and therefore more persistent in their effect. As additional evidence Kargapolova (1937) found that catechol and hydroquinone were the most toxic of a number of

simple phenols which were tested against the spores of the rust fungus.

It may well be that catechol-tannins are the most toxic against the wheat rust and the white pine blister rust, but from this it does not follow that ~~it is~~ they are the most toxic against all fungi. The complexity and varied chemistry of the tannins combined with the complexity of the enzymatic systems of the fungi, leaves plenty of scope for differential toxicity of a certain type of tannin against different fungi or for variations in the behavior of different tannins against the same fungus.

Our knowledge regarding the enzymatic breakdown of tannins is very incomplete at present. It has long been known that various fungi produce an enzyme, the so-called tannase, which can split off gallic acid from a solution of the gallotannic acid from oak galls. Rippel and Keseling (1931) studied the production of tannase from a number of fungi. They found that while the production of tannase occurred only in presence of tannin it was not necessarily associated with the ability to utilize tannin. Of the fungi which were tested, Penicillium, Citromyces and Aspergillus spp. were the only ones which could use the compound as the sole source of carbon.

Tannase is an enzyme complex of varied constitution. The tannase of a certain fungus may be able to decompose only a certain type of tannin as shown by the work of Fuller and Nierenstein (1944). They found that whereas Polyporus

spp. decomposed only pyrogallol-tannin, Collybia spp. decomposed the catechol-tannin, and only this type of tannin could be found in its hyphae. Nierenstein isolated the enzymes from the two fungi and named them according to the type of tannins they decomposed, pyrogallase and catecholase.

Freudenberg and Walpuski (1921) studied the decomposition of tannins of European chestnut by Aspergillus-tannase and obtained traces of gallic acid, ellagic acid and sugars, largely glucose. No information is available concerning the effect of tannase from other fungi on chestnut tannin and we do not know what the tannase decomposition of the tannins of American, Japanese and Chinese chestnut may yield. Nor do we know the type of enzymes which are produced by Endothia. The above discussion does show, however, that a pronounced specificity exists in the interaction between the tannase system of a fungus and the tannins which it can break down.

Until much additional information is available it is impossible to explain the effect of the tannins derived from the bark of Chinese chestnut on the one hand and those from Japanese and American chestnut on the other. Pending more complete data the following is suggested as a working hypothesis:

The tannase complex of E. parasitica decomposes pyrogallol-tannins and in doing so produces a simple phenol which is toxic to the fungus. The tannins in the bark of Chinese chestnut belong to the pyrogallol group, whereas the barks of American and Japanese chestnut contain a

tannin which is a mixture of pyrogallol- and catechol-tannins. In media where the overall concentration of tannin is the same, the concentration of the active ingredient may be considerably lower in the media containing the extract from the bark of Japanese and American chestnut and their toxicity, therefore similarly reduced.

It is possible that the phenol which is the actual cause of toxicity is pyrogallol. This is a likely breakdown product of pyrogallol-tannin, and the compound is, as evidenced from the assay with the simple phenols, extremely toxic to the fungus.

It has been mentioned that the fungus readily penetrates the sapwood, in which the tannins primarily belong to the pyrogallol-group. This apparently contradicts the suggested hypothesis. But, the tannin content of sapwood is very low. Thus, Nierenstein (1934) mentions that the sapwood contains 0.36 per cent as compared to 4.0 per cent in the heartwood of Gleditsia, and Frey and Leinback (1925) state that the tannin content in the sapwood of oak trees is "very low", and expect the same to be true in C. dentata. Apparently the content is so low that it has little or no toxic effect on the fungus.

Disease Resistance in Chestnut. A Summary

To bring together in a complete account the information which has been obtained from this study concerning the role of tannins in the resistance of chestnut to E. parasitica,

the following hypothesis is suggested:

The tannins in chestnut are present either in a bound form as an addition compound with some other colloid, in which case their solubility depends on how closely the two colloids are bound together, or they are present partly as bound and partly as free tannins, their solubility depending on the relative abundance of free tannins which are readily soluble and bound tannins which are more or less insoluble.

The quality of the tannins in the bark differs from one species to another. In Japanese and American chestnut, they are a mixture of catechol- and pyrogallol-tannin, whereas in the Chinese species only pyrogallol-tannins are found. Of these two, pyrogallol-tannins are the ones which are toxic to the fungus. By the action of its tannase complex it decomposes the tannins to simple phenols, possibly among others to pyrogallol, which is toxic to the fungus.

The toxicity of the tannins of the host plant will depend on the amounts in which they are present in an active condition, i.e. their solubility and their qualitative characteristics.

The tannins of American chestnut are the least soluble of the three tannins and those of the Chinese chestnut are more soluble than those of the Japanese species; consequently the Chinese chestnut is the most resistant, the American chestnut is the most susceptible and Japanese chestnut occupies an intermediate position between the two.

As an additional factor of resistance the tannins of

the bark of Chinese chestnut are pyrogallol-tannin and therefore more toxic than in the two other species, where they are mixtures.

Further study will be needed to test this hypothesis. The solubility of tannins of the three species should be studied in detail, using an extraction technique as described by Huber, and the condition of the tannins in the plant cell examined microscopically. The chemistry of the tannins should be determined as accurately as possible and their enzymatic breakdown by the fungus elucidated.

The results of the experiments performed by the writer should not be interpreted to mean that the tannins are the only factors of resistance to the Endothia. It is possible, as mentioned, that other compounds, perhaps coloring matter, is responsible for part of the chemical resistance.

The structure of the bark should also be considered. Wound cork formation may be a factor of importance either because it is formed more abundantly or because its structure is more efficient in checking the advancing mycelial mat. To clarify this a comparative study should be made of the healthy and diseased bark of the three species of the chestnut. This study should not be limited to the anatomy of the bark; chemical changes which take place in the bark when it is entered by the fungus should be investigated and special attention should be paid to the behavior of the tannins in the diseased bark.

The Determination of the Relative Resistance of Hybrid Seedlings

The above study seems to focus the attention on two possible ways in which the resistance of hybrid seedlings could be determined:

1. Determine the type of tannins present in the bark of the seedlings by means of the formaldehyde-hydrochloric acid test.
2. Determine the solubility of tannins in the bark of the seedlings.

The qualitative test is perhaps the most promising; the technique is fairly simple and only a small amount of plant material would be required.

We do not at present have enough proof that the quality of the tannins is actually a factor in resistance of chestnut, but the evidence is sufficiently pertinent to make further study seem worth while. The first logical step would be to determine the quality of the tannins in a number of older hybrids, of which the resistance is known. If this study substantiates the hypothesis, it seems that the test can be applied safely to young seedlings.

The solubility will be harder to determine. The hide-powder method is not satisfactory for a study of this nature, because the powder would have to be made up fresh repeatedly and the technique is altogether too time consuming. It will probably be necessary to resort to some titration analysis. Also, in this case the study should begin with the study of

the condition in old trees.

The study of the tannins presented here emphasizes the complexity of the whole problem of resistance. Considering this, it appears that it may be dangerous to draw any conclusions from a test which only determines one of the factors involved. The further studies which have been suggested will undoubtedly clarify this problem; here all that can be done is to point out the danger of a misinterpretation of the results from a test which has been oversimplified.

NUTRIENT EXPERIMENTS

Disease endurance of seedlings as affected by three different concentrations of nutrients

Preliminary Experiments

On December 13, 1948 a small perliminary experiment was set up to determine the effect of nutrient levels and balances on the susceptibility of C. mollissima, C. crenata and C. sativa. The last species is, as previously mentioned, comparable to C. dentata in its susceptibility.

Seedlings which had just finished their first growing season were taken into the greenhouse in the middle of November and planted in builders' sand in glazed crocks, four plants to a crock.

Considering the work of Diller et al., (1946) it was decided to try nutrients with different balances of potassium and nitrogen, and, in addition to try complete nutrient solutions at two concentrations: (a) the usual concentration, and, (b) 1/10 the usual concentration.

The so-called Purdue F solution was used, the formula being as follows (Withrow 1943):

Compound	Grams per 50 gallon
$MgSO_4$	26
$Ca(H_2PO_4)_2$	31
$CaSO_4$	152
KCl	80
KNO_3	110
$(NH_4)_2SO_4$	28

To this was added 8.3 ml. of the following trace-element solution for each gallon of the nutrient solution of full concentration; and 1/10 of this amount added to

each gallon for the weaker nutrient solution.

Ferric tartrate	7.5 g.
Boric acid	0.5 g.
Copper sulfate	0.5 g.
$KMnO_4$	0.75 g.
Water	500 ml.

Four modified solutions were used and designated as -K, -N, +K and +N. They can be described as follows:

-K: K omitted from the complete solution.

-N: Containing 16.5 per cent of the N in the complete solution.

+K: 30 per cent K added.

+N: 20 per cent N added.

The application of the nutrient solution was started December 12, 1948 after the plants had resumed growth, 25 ml. being applied to each crock every other day, three times a week. Each treatment was applied to 4 plants, one of which was a control.

After two months, on February 11, 1949, the plants were inoculated with the fungus. The inoculation technique can be described as follows: A cut was made on the stem about 2 inches above the root collar, lifting the bark from the wood on an area about 1/8 inch wide and 1/2 inch long. The inoculum was an agar block cut from a culture of the fungus, which was about two weeks old and had numerous spore pustules on the surface. The piece was placed in the wound with the spore pustules facing the wood, the bark was returned in place, and the area covered with a piece of cotton fastened by scotch tape.

Data were collected March 5, April 6, and May 13, but only the data from the last observation have been shown in TABLE 13 below.

TABLE 13. DISEASE CONDITION OF CHESTNUT TREATED WITH DIFFERENT CONCENTRATIONS AND BALANCES OF NUTRIENT SOLUTIONS

Solution	Number of Plants in the Different Classes of Disease Development		
	Chinese Chestnut	Japanese Chestnut	European Chestnut
Complete	3 none h	3 none h	1 none h 1 light 1 dead
1/10 complete	2 medium 1 heavy	2 medium 1 heavy	1 heavy 2 dead
+N	2 none h 1 light	1 none h 1 light 1 heavy	1 none h 1 light 1 heavy
-N	2 none h 1 light	1 none h 2 medium	2 medium 1 dead
+K	3 none h	3 none h	2 medium 1 dead
-K	3 none h	3 none h	1 none h 1 heavy

Explanation: Disease condition was classified as follows:

None = No spread of fungus
Light = Slow spread of fungus
Medium = Fungus spreading, but resistance expressed in the formation of hypertrophied stem tissue.
Heavy = Fungus spreading, no apparent resistance
h = indicates that the wound is healed as was the case on all the control plants, on which the incision was made without inoculation.

The data seem to indicate that a high degree of susceptibility to disease is correlated to the low nutrient level; thus Chinese and Japanese seedlings growing in a full normal nutrient solution were all healed at the end of the experiment, whereas the plants of both species kept at the low nutritional level (1/10 of normal) showed two medium and one heavily attacked plants. The results are similar for European chestnut with two dead and one heavily attacked at 1/10 the full concentration, as opposed to one healed, one light attack and one dead plant in the solution of full concentration. The data do not indicate any effect of the changes in the balance of the solution.

With this information it was decided to concentrate the efforts of the main experiment on the effects of different concentrations of the balanced nutrient solutions. Furthermore, it was decided to investigate the possible effect of plant metabolites on the susceptibility. It has already been mentioned that there is a correlation between disease development and the carbohydrate content of the plant in the case of the seedling blight of wheat and corn (Dickson et al., 1923). In the work with Dutch elm disease it has been found that susceptibility increases when the content of reducing sugars decreases. Therefore, it was thought worthwhile to investigate the problem with Endothia parasitica.

The Main Experiment
Material and methods

Mineral Nutrition

The experiment was designed to obtain the following information (1) the relative susceptibility of C. dentata, C. crenata, and C. mollissima, (2) the effect of concentration of the nutrient solution on susceptibility and (3) the effect of the length of time in which the plants have been growing under the different nutritional conditions.

(1). The plants were obtained from nuts gathered in October 1948 and planted immediately in the greenhouse. The Chinese and Japanese nuts were gathered from trees growing in the Hamden plantation. They were open pollinated nuts and part of them may very well be of hybrid origin. This is a drawback in the experiment, which, however, could not be avoided because of the mixed nature of the plantation.

The American nuts were collected from trees growing near Roxbury, Connecticut, in a place where no hybridization could occur.

(2). The Purdue P solution was used as in the preliminary experiment, but applied in the following concentrations: Full strength, 1/3, and 1/9 of full strength. As in the preliminary experiment, it was applied at the rate of 25 ml. per crock (3 plants) every second day, 3 times a week.

(3). The first plants were transplanted to the crocks on April 1, and received the first nutrient application on April 4. The nut with what remained of the cotyledons was

removed from all plants at the time of transplanting. Three plants were placed in each crock instead of 4 as in the preliminary experiment. Builders' sand was used as the medium.

A second series was transplanted May 14 and received the first nutrient application two days later.

All plants were inoculated on June 15 and 16 after the first plants had been treated with nutrients for 72 days and the second series for 30 days.

Application of the nutrient solution was continued to the end of the experiment.

Plant Metabolites

This problem was approached in two ways. (1) The plants were girdled by tying raffia tightly around the stem 3 - 4 inches above the root collar. As the plants grew, this became a very effective girdle which prevented the downward movement of assimilation products beyond the girdle. This was evidenced by a pronounced swelling of the stem above the point of strangulation.

(2) The pots were covered with hoods of black cloth supported by a wire frame. This prevented CO_2 assimilation and after some time the plants lost their leaves; later a set of etiolated leaves appeared.

Both series were watered with nutrient solution of full strength every other day, three times a week and the effect of length of treatment was considered as mentioned

above. Only American and Japanese chestnut was used in this part of the experiment, as the germination percentage of the Chinese nuts unfortunately was too low to yield a sufficient number of plants.

General Layout of the Experiment

The experiment was set up as five randomized blocks with one crock for each treatment or 26 crocks in each block. This gave a total of 15 plants under each treatment.

Collection of Data

All plants were measured to the nearest 1/4 inch at the time of transplanting and thereafter once a month, the last time on August 15. At each time of measurement the number of leaves per plant was taken, and notes were made regarding the general appearance of the plants.

Data concerning the development of disease were first taken July 11, four weeks after inoculation and thereafter at two-week intervals; the experiment terminated after collection of the data on August 22.

Five classes of disease development were considered and were based on the amount of mycelium which could be seen as an orange discoloration under the bark:

1. None. No spread of fungus.
2. Low. Hyphae not advanced more than 1 mm. from edge of wound.
3. Medium. More than "low", but stem not half girdled by the fungus.

4. High. Stem more than half girdled.

5. Plant dead.

Numerically the 5 classes were rated as 1, 2, 3, 4 and 5 respectively. The data have been computed on a pot basis (three plants). Thus, one plant with "medium" and two with "high" disease development would give the pot a rating of 11.

On August 22 when the last readings were taken, the plants were cut off at the ground level, were stripped for the leaves, and the stems allowed to dry and finally subjected to an analysis for the following elements: K, Ca, Mg, P, Mn, Fe, Al, Zn, Na, Cu and B.

Results

General Results

A total of 450 inoculations was made; of these only 386 could be accounted for at the end of the experiment, as the 60 plants growing in darkness died because of the treatment. An attempt to save these plants by exposing them to alternate light and dark rather than total darkness, failed. In addition four plants had died during the experiment of causes other than the inoculations, thus bringing the number to 386.

TABLE 14 shows the over-all result of the inoculations of the plants subjected to treatments of nutrients of different strengths for different lengths of time. The table emphasizes the pronounced variation which occurred within species and treatments.

The results of the inoculations have been summarized in TABLE 15.

TABLE 15. RESULTS OF INOCULATIONS ON THREE SPECIES OF CHESTNUT, 68 DAYS AFTER TREATMENT

Species	Number positive	Percentage of species total	Number negative	Percentage of species total	Species total
Chinese	59	67.05	29	32.95	88
Japanese	97	65.10	52	34.90	149
American	135	90.60	14	9.40	149
	291	75.39	95	24.61	386

The data presented here are too condensed to be of any real value in the interpretation of the results of the experiment; they do, however, emphasize how well the inoculation technique worked, 90.60 per cent successful on the susceptible species. The 9.40 per cent in which infection did not occur probably failed for reasons other than a factor of inherited resistance. The lower percentage of successful inoculation on the Chinese (67.05 per cent) and Japanese chestnut (65.10 per cent), on the other hand, is clearly a result of the inherited resistance in these species. This difference between the species will be better shown in the following presentation.

The Effect of Nutrient Treatments on Disease Development

In TABLE 16 the average disease rating has been arranged

by species, treatments and dates of recording.

TABLE 16. THE AVERAGE DISEASE DEVELOPMENT OF E. PARASITICA ON THREE SPECIES OF CASTANEA, AS AFFECTED BY STRENGTH OF NUTRIENT SOLUTION AND THE LENGTH OF TIME WHICH THE PLANTS HAD BEEN SUPPLIED WITH NUTRIENT PRIOR TO INOCULATION

		72 days treatment			30 days treatment		
		Concentration of nutrient solution					
Date	Species	1	1/3	1/9	1	1/3	1/9
7/11	Chinese	3.8	5.0	5.4	3.6	3.6	3.8
	Japanese	3.8	5.0	5.8	3.0	5.0	4.2
	American	4.6	6.6	6.0	5.2	6.0	5.0
7/26	Chinese	5.1	5.4	6.8	3.6	4.9	4.8
	Japanese	5.0	5.8	7.2	4.8	4.0	5.8
	American	7.6	8.6	8.6	6.8	6.6	7.0
8/15	Chinese	6.6	6.8	7.6	4.0	5.0	5.4
	Japanese	5.8	7.0	7.6	4.8	7.1	6.6
	American	10.6	10.6	10.6	11.0	11.0	9.0
8/22	Chinese	7.0	7.0	8.8	5.4	6.0	6.0
	Japanese	6.0	7.2	8.0	5.0	8.0	7.0
	American	11.6	11.6	10.8	11.0	11.4	9.8

The values represent disease rating, they are on a crock basis and are the average for 5 crocks distributed at random in 5 blocks.

The analysis of variance is condensed in TABLE 17.

TABLE 17. ANALYSIS OF VARIANCE FOR THE EXPERIMENT ON EFFECT OF NUTRIENT LEVELS ARRANGED BY DATES OF RECORDING

Source of Variation	Degrees of Freedom	F Values Dates of recording 7/11	7/26	8/15	8/22
Blocks	4	4.20**	7.22**	7.15**	6.98** 1)
Length of treatment	1	5.89*	4.25*	6.52*	6.06*
Concentration of nutrient	2	6.17**	4.49**	1.53	1.77
Species	2	7.48**	22.91**	51.18**	53.02**
Length x Conc.	2	1.26	1.96	0.75	1.24
Length x Species	2)				
Conc. x Species	4)	0.50	0.91	1.24	1.23
LengthxConc.x Species	4)				
		Mean squares			
Error	68	2.18	3.39	3.61	3.39
Correction from the mean	1				
Total	89 / 90				

The J.S.D. ("Just Significant Differences") which have been listed in subsequent tables are based on the above values for the mean squares of the error term.

1) ** significant at the 1 per cent level.
   * significant at the 5 per cent level.

The analysis emphasizes the difference in disease development on the three species. In order to facilitate the interpretation the averages, regardless of treatments, have been computed below and presented with the corresponding "just significant differences".

TABLE 18. THE AVERAGE DISEASE DEVELOPMENT ON THREE SPECIES OF CASTANEA INOCULATED JUNE 15-16 WITH E. PARASITICA

Species	Date of Recordings			
	7/11	7/26	8/15	8/22
American	5.57	8.07	10.47	11.03
Japanese	4.47	5.87	6.50	6.93
Chinese	4.17	4.93	5.90	6.67
J.S.D. .05	.760	.948	.978	.948
J.S.D. .01	1.009	1.259	1.299	1.259

The averages are computed on the basis of a crock containing three plants.

This shows the difference between the native species on the one hand and the two Asiatic species on the other. The difference was significant at the one-per cent level from the very first day of recording, and increased in magnitude as the experiment progressed.

At no time of the experiment did the difference between Chinese and Japanese chestnut reach a level of statistical

significance, although the amount of attack was slightly larger on the Japanese seedlings throughout the period.

From TABLE 19 it can be seen that the effect of the concentration of the nutrient solution attained significance only at the two first dates of recording.

There was no difference between the 1/3 and 1/9 concentration of nutrient solution, but both these concentrations resulted in a degree of disease development which, in the early part of the experiment, was more severe than on the plants grown in full-strength solution. Two months after inoculation the difference ceased to be significant.

TABLE 19. THE AVERAGE DISEASE DEVELOPMENT ON SEEDLINGS OF CASTANEA INOCULATED JUNE 15-16 WITH E. PARASITICA, AND GROWING ON THREE DIFFERENT NUTRITIONAL LEVELS

Concentration of Nutrient solutions	Date of recording			
	7/11	7/26	8/15	8/22
1	3.97	5.47	7.10	7.70
1/3	5.20	6.70	7.80	8.40
1/9	5.03	6.70	7.93	8.53
J.S.D. .05	.760	.948	.978	.948
J.S.D. .01	1.009	1.259	1.299	1.259

The averages are computed on the basis of a crock containing three plants.

There is some evidence to indicate that this early difference is due to a delayed penetration of the fungus in the plants growing in the full nutrient solution rather than to a slower spread of the fungus once it has penetrated into the host. Thus, although 64 of the plants growing in the full-strength solution showed no sign of the disease on July 11 the corresponding value for the two other concentrations is 43 in both cases. The distribution of the remaining diseased plants in disease-development classes 2, 3, 4 and 5 is very similar for all three concentrations as shown in TABLE 20.

TABLE 20. NUMBER OF PLANTS IN DISEASE DEVELOPMENT CLASSES 2, 3, 4 AND 5, EXPRESSED AS A PERCENTAGE OF THE TOTAL NUMBER OF PLANTS IN THESE CLASSES.

Classes of disease development	Concentration of Nutrient Solution					
	1/9		1/3		1	
	7/11	7/26	7/11	7/26	7/11	7/26
2. Low	73.91	41.67	65.21	38.59	76.00	48.85
3. Medium	19.57	33.33	26.09	35.09	16.00	27.91
4. High	6.52	25.00	8.70	21.06	8.00	20.93
5. Dead	...	...	...	5.26	...	2.33
Total number of plants	46	60	46	57	25	43

If the growth rate was slower in the full-strength solution it would mean that the number of plants, expressed

as a percentage, in class 2 and 3 should be larger than for the two lower concentrations. This does not seem to be the case as indicated by the TABLE 20.

The Effect of Length of Treatment

The effect of the length of treatment was significant at the 5 per cent level throughout the experiment, as shown in TABLE 21.

TABLE 21. THE EFFECT OF DIFFERENT LENGTHS OF TREATMENT WITH NUTRIENT SOLUTION ON THE SUSCEPTIBILITY OF CASTANEA TO E. PARASITICA

Length of treatment	Date of recording			
	7/11	7/26	8/15	8/22
	Disease ratings			
72 days	5.11	6.69	8.13	8.69
30 days	4.36	5.67	7.11	7.73
J.S.D. .05	.760	.774	.779	.774
J.S.D. .01	1.009	1.028	1.061	1.028

The plants, which had been treated 72 days with nutrients before inoculation, were more severely attacked than those which had been treated for the shorter length of time.

The Effect of Nutrient Treatments on the Growth of Chestnut Seedlings

The effect of the treatments on seedling growth is

shown in TABLE 22 which presents the analysis of variance of the growth increment between May 20 and August 15, that is, from the time the treatments were begun on the plants receiving the 30-day treatment until the end of the experiment, a period of 87 days.

TABLE 22. ANALYSIS OF VARIANCE OF GROWTH INCREMENT BETWEEN MAY 20 AND AUGUST 15. THE ANALYSIS IS BASED ON THE AVERAGE HEIGHTS OF 3 PLANTS IN EACH CROCK

Source	Degrees of Freedom	Sum Squares	Mean Squares	F Value
Blocks	4	20.04	5.01	2.19
Length of treatment	1	31.69	31.69	13.84xx 1)
Conc. of nutrient	2	7.00	3.50	1.53
Species	2	27.90	13.95	6.09xx
Length x Conc.	2	.51	.26	.01
Length x Species	2			
Conc. x Species	4	13.08	1.31	.57
Length x Species x Var.	4			
Error	68	155.71	2.29	
Total	89	255.93		
Correction for mean	1			
n	90			

1) xx significant at 1 per cent level.

The length of treatment had a significant effect; the increment being larger in the plants which had only been treated 30 days with a nutrient solution at the time of inoculation, than in the plants which had been treated for 72 days. This effect is shown in the tables below.

TABLE 23. AVERAGE HEIGHT IN INCHES OF CHESTNUT SEEDLINGS TO WHICH NUTRIENT HAD BEEN APPLIED FOR DIFFERENT LENGTHS OF TIME

Length of treatment	Dates of recording						Average increment from 5/20 to 8/15
	4/4	4/28	5/20	6/16	7/13	8/15	
72 days	8.51	8.91	9.09	9.16	9.37	9.66	0.57
30 days			8.37	8.63	9.48	10.12	1.75

The average height of the plants as it was expressed by the three species and as it was affected by the strength of nutrient has been computed in TABLE 24.

TABLE 24. AVERAGE HEIGHT IN INCHES COMPUTED ON THE BASIS OF (1) SPECIES AND (2) THE STRENGTH OF THE NUTRIENT WITH WHICH THEY WERE TREATED

Dates of Recording	Chestnut Species					
	Chinese		Japanese		American	
	Length of treatments					
	72	30	72	30	72	30
4/4	9.16		10.99		5.38	
4/28	9.32		11.90		5.52	
5/20	9.41	8.97	12.28	10.95	5.60	5.18
6/16	9.45	9.10	12.40	11.41	5.62	5.07
7/13	9.56	9.96	12.92	12.62	5.60	5.85
8/15	9.81	10.21	13.35	13.77	5.83	6.39
Average Increment from 5/20 to 8/15	0.40	1.24	1.07	2.82	0.23	1.21

Dates of Recording	Concentration of nutrient solutions					
	1/9		1/3		1	
	72	30	72	30	72	30
4/4	8.39		8.45		8.69	
4/28	8.75		8.83		9.16	
5/20	8.84	8.07	9.18	8.18	9.26	8.85
6/16	8.96	8.48	9.22	8.34	9.30	9.06
7/13	9.23	9.15	9.33	9.10	9.55	10.17
8/15	9.26	9.60	9.62	9.67	10.12	11.11
Average Increment from 5/20 to 8/15	0.42	1.53	0.44	1.49	0.86	2.26

The difference in the growth increment of the three species is statistically significant but is hardly important in connection with the scope of the experiment.

From the analysis of variance it appears that the concentration of nutrients had no significant effect on the increment. This may seem peculiar in view of the values for the total increment between May 20 and August 15 shown on the last line in TABLE 24. Here the increment resulting from the full-strength solution is 50-100 per cent larger than that produced by seedlings receiving the two weaker solutions. This apparent discrepancy between the two tables is undoubtedly due to the extreme variation within treatment which would bring down the F value. The writer feels that had it been possible to work with larger populations it would have been possible to show a significant effect of the treatments.

A discoloration of the leaves developed early in the life of the plants before they were subjected to any treatment. It was of general occurrence throughout the experiment and although the data concerning this feature have not been analyzed statistically it is quite clear that no difference appeared which could be ascribed to species or treatment. The cause was not discovered. It was not caused by either fungi or insects, and as it occurred prior to the treatment with nutrients it could not be due to an application in excess. Also a deficiency in some element seemed unlikely. The discoloration was first attributed to sun

scald, but the idea was dismissed as it reoccurred on new leaves after the greenhouse had been heavily white-washed. The possibility remains that the condition was caused by the Nico-fumes which were used in the greenhouse for fumigation.

The Chemical Analysis

In the chemical analysis no attempt was made to maintain the statistical design of the experiment. The plants were lumped together according to species and treatment, disregarding the five blocks. The results are presented below in TABLE 25. The writer is indebted to the Department of Analytical Chemistry of The Connecticut Agricultural Experiment Station for the actual analysis.

The content of the different elements in the various species grown at different nutritional levels may be summarized as follows:

K : No apparent difference between species and no effect of the different treatments.

Ca: The content varies considerably between species and between treatments within species. There does not seem to be any correlation between the Ca content and the strength of nutrient solution. There is, however, some indication that the shorter period of application resulted in a smaller Ca content on a percentage basis, and that it is larger in the American than in the Chinese and Japanese chestnut seedlings.

Mg: No apparent effect of the treatments or difference between the species.

P : No apparent effect of the treatments or difference between species.

Mn: The manganese content seems to be slightly higher in the seedlings of Chinese chestnut. The three species do not react in the same way to the concentration of nutrient. The seedlings of Chinese and American chestnut seem to have responded to the treatment, showing an increasing Mn content the lower the concentration of nutrient applied. The seedlings of Japanese chestnut show no effect of the concentration of the nutrient.

Fe: The iron content does not seem to have been affected by the different concentrations of nutrient solution and seem to be similar in the three species. It does seem as if the plants on the shorter treatment have a lower content of this mineral, but the difference is not very clear cut.

Na, Cu and B: The concentration of these elements appears to be the same regardless of species and treatments.

Al and Zn: A variation in the content of these two elements due to the treatments is not apparent, but the seedlings of American chestnut seem to have a slightly higher content. The difference, however, is not very pronounced.

Unfortunately the nitrogen content of the seedlings

was not determined. It would have been very interesting to have these values, but by mistake they were not procured by the chemist who carried out the analysis.

Results from Experiments with Plants Grown in Darkness and from Girdled Plants

Complete darkness was, as mentioned previously, too drastic. The plants died so early in the course of the experiment that it was impossible to obtain any information regarding the effects of the treatment on disease development. An attempt was made to save the plants by exposing them to alternate light and darkness, but the change was apparently started too late to be effective.

If similar experiments are tried at a later date, it will be necessary to reduce the light source rather than completely eliminating it.

The results of the girdling experiment are shown in TABLE 26.

TABLE 26. THE EFFECT OF GIRDLING ON THE DEVELOPMENT OF DISEASE IN JAPANESE AND AMERICAN CHESTNUT SEEDLINGS INOCULATED WITH E. PARASITICA

		72 days treatment			30 days treatment		
			Position of inoculation			Position of inoculation	
Date	Species	Control	Upper	Lower	Control	Upper	Lower
			Disease rating				
7/11	Japanese	3.8	5.0	4.6	3.0	4.4	4.4
	American	4.6	7.0	6.6	5.2	5.2	5.8
7/26	Japanese	5.0	5.6	5.0	4.8	4.6	5.0
	American	7.6	10.2	8.0	6.8	8.0	8.2
8/15	Japanese	5.8	6.0	5.8	4.8	5.2	6.0
	American	10.6	12.4	11.4	11.0	10.4	9.6
8/22	Japanese	6.0	6.6	6.0	5.0	5.4	6.2
	American	11.6	12.8	12.4	11.0	10.8	10.0

The "controls" in the above experiment consist of plants which were treated with full-strength nutrient solution. The values are on a pot basis and are the average of five pots in five randomized blocks.

The analysis of variance is given in TABLE 27 below.

TABLE 27. ANALYSIS OF VARIANCE FOR THE GIRDLING EXPERIMENT ARRANGED BY DATES OF RECORDING

Source of Variation	Degrees of Freedom	F Values: Dates of recording 7/11	7/26	8/15	8/22
Blocks	4	2.85*	3.20*	2.06	3.65* [1]
Species	1	11.60**	27.12**	75.11**	102.61**
Treatment	2	3.30*	1.02	0.19	0.88
Length of treatment	1	1.78	1.23	1.86	4.51*
Length x treatment	2	0.51	0.69	0.27	.17
Species x treatment	2				
Species x length	1	0.33	0.31	.47	.49
Species x length x treatment	2				
		Mean squares			
Error	44	3.04	5.43	5.61	4.53
Total	59				
Correction	1				
	60				

1) ** significant at the 1 per cent level.
* significant at the 5 per cent level.

The difference in resistance between the two species is again very strongly expressed, but the effect of the treatments are not clearly shown. On the first day of the record (7/11) the difference (see TABLE 27) between the control on the one hand and the girdled plants on the other

is significant at the 5 per cent level; this difference, however, is not significant on later recordings. The difference between upper and lower inoculations did not reach a significant level.

The length of treatment showed a significant effect only on the last day of the record, when the plants which had been treated the shortest time with nutrient solution showed the least amount of disease development (see TABLE 27) but considering that the first three recordings showed no significant difference, the difference on the last day probably should be taken with some reservation.

The increment of the plants is similar to that of the plants in the nutrient experiment. The analysis of variance of the increment between 5/20 and 8/15 has been shown below.

TABLE 28. THE ANALYSIS OF VARIANCE FOR GROWTH INCREMENT FROM THE EXPERIMENT WITH GIRDLED SEEDLINGS OF JAPANESE AND AMERICAN CHESTNUT

Source of Variation	Degrees of Freedom	Sum Squares	Mean Squares	F Value
Blocks	4	12.6052	3.1513	1.45
Species	1	25.3606	25.3606	11.66** 1)
Length of treatment	1	12.6052	12.6052	5.79*
Treatment	1	8.6769	8.6769	3.99
Length x treatment	1	1.3951	1.3951	.64
Species x Length	1)			
Species x Treatment	1) 3	6.4931	2.1644	.99
Species x Treatment x Length	1)			
Error	28	60.9254	2.1759	
Total	39	128.0615		
Correction	1			
	40			

1) ** significance at the 1 per cent level.
* significance at the 5 per cent level.

The difference between the species and between the two lengths of treatments were significant at the one and five per cent levels, respectively, but the increment did not show a response to the girdling.

For the chemical analysis the parts of the girdled

plants above and below the girdle were considered separately.

The result of the analysis has been shown in TABLE 29.

The results are in many respects similar to those already presented for the nutrient experiment. Thus, the Ca content is somewhat higher in the seedlings of American chestnut, than in the Japanese chestnut seedlings, and it is lower in the seedlings which had only been treated 30 days with nutrient prior to inoculation.

Also, K, Mg, P, Fe, Na, Cu and B show percentages similar to those found in the nutrient experiment. It should, however, be mentioned that some of the values for the girdled seedlings of American chestnut on the longest treatment are out of line with the other results, being considerably larger.

In other respects there is no apparent difference between the species or treatments.

Manganese, aluminum, zinc and calcium are not evenly distributed in the plant; and in the cases of Ca, Mn, and Zn, the content is higher above the girdled point, while for Al the largest concentration is found below the girdle. These differences, however, are probably due to a differential uptake of different parts of the plant rather than a stoppage of conduction by the girdle. It seems reasonable to assume that the girdle would not be able to prevent some ions from passing while others were allowed to pass through. Had the girdle been able to prevent ions from passing it

would mean that all values from above the girdle should be similarly lowered.

Discussion

The Nutrient Experiment

The results exemplify how factors of the environment and factors of resistance interact to bring about the actual relative susceptibility.

The data cannot be explained simply on the basis of a concentration effect, the susceptibility increasing, the lower the concentration of the nutrient applied. Such an explanation would fail to account for the fact that the difference between concentration treatments was significant only at the two first dates of recording. Furthermore, it would be in disagreement with the results reported by Diller (1949) who found that the application of a liquid fertilizer, apparently a standard Hoagland's solution, resulted in increasing susceptibility of young seedlings grown under greenhouse conditions.

If the penetration and the further growth of the fungus is considered separately as two stages in the development of the disease, it is found that they represent two distinct effects of the concentration treatments.

Data were presented in TABLE 20 which show that the low average disease-development rating on June 11 and June 26 for the full-strength solution was due to a smaller number of plants showing signs of the disease, rather than a

slower spread of the fungus, once it had become established in the host. It is not possible, because of the way in which the disease was observed, to make a distinction between the actual penetration of the fungus into living host tissue, and the early growth until the infection can be observed by the naked eye. All that can be said with certainty is that after the disease has reached an early, but undefined, stage of development in the host, new factors come into effect (or perhaps some factor is eliminated) and drastically change the effect of concentration-treatments. Thus, whereas the plants on the full strength solution were less susceptible than the plants on lower concentrations in the early part of infection, there is some indication that they were slightly more susceptible than these plants after the first four weeks. This can be seen from TABLE 19 which only gives similar values for the treatments with different concentration of nutrient solution.

The effects of the concentration of the nutrient solution were the same regardless of how long they had been applied prior to the inoculation, as shown by the non-significant interaction terms (length x concentration) in the analysis of variance in TABLE 17.

Length of treatment itself had a significant effect; the plants on the shortest treatment being the least susceptible throughout the experiment.

It is impossible from the results of this experiment to draw any definite conclusion regarding the type of reactions,

which are involved in the treatment effects mentioned above. However, the following working hypothesis is suggested. Suppose that the two stages in the disease development which respond differently to the treatments with different concentrations of nutrient solution are (1) the actual penetration from the wound into the living host tissue and (2) the further spread of the fungus.

The penetration is brought about by a mass action of the hyphae, as previously mentioned. A certain amount of hyphae must develop before penetration takes place. During their formation in the wound the plant will be walling off the wounded area, forming a layer of wound cork. The relative speed with which these two processes take place will determine the time of the penetration. The concentration of nutrient could conceivably influence the rapidity with which a new phellogen and eventually a layer of wound cork is formed.

Then the result of the experiment can be explained as follows: In the plants growing on the full nutrient solution the wound cork formation is more rapid (or the cell walls perhaps thicker) than with the lower concentrations. The wound cork will act as a temporary barrier against the fungus, and penetration will be delayed.

Having once entered the living host tissue, the hyphae will be under the influence of a new set of factors which in turn may respond differently to the concentration of

the nutrient solution. A possible explanation would be that an element essential to the growth of the fungus may be present in a critical concentration; the plants growing on the full-strength nutrient solution would therefore be more susceptible. The reaction would be similar to that suggested by Walker and Kendrick (1948) and discussed previously, but it would be the plant which was winning out in the competition for the element and not the fungus.

The result of the chemical analysis may be suggestive in this connection, but the chemicals in the living plant may be present in an inert condition in which case they have no influence on the fungal growth, and the data will never be a proof one way or the other. Actually, as mentioned previously the result showed little or no effect of the treatments.

Another weakness in this hypothesis is that it fails to explain the lower susceptibility of the plants on the 30-day treatment. This effect, however, may be the result of an altogether different set of factors, as discussed later.

Another possible explanation may be found in the suggestion by Diller et al., (1946) that the chloride ion has a toxic effect on the chestnut seedlings. The plants grown on the strongest solution would show the most vigorous reaction to the toxin and consequently be the most susceptible. There is no evidence from the results actually proving this hypothesis. It has one advantage, however, over the

hypothesis based on the lack of some essential element in the growth of the fungus; it can explain the lower susceptibility of the plants which had been treated with nutrient for only 30 days prior to inoculation. These plants had been exposed for a shorter time to the alleged toxic effects and would be less susceptible.

The difference in susceptibility of the plants exposed to the two treatment lengths cannot be expected to be of a permanent nature, but would equalize as the relative difference in length of treatments decreases.

Other explanations of the effects of the length of treatment are possible. For example: remaining undisturbed on the germination bench for a longer time the chestnut seedlings may have developed a larger root system which enabled them to overcome the shock of transplanting better. The better growth of these plants (TABLE 23) may favor this explanation. Another possibility would be that the plants subjected the shorter treatment were brought into the experiment at a time when they were exposed to a different and more favorable set of environmental conditions which enabled them to withstand the inoculations better for some time. Both these explanations bring in a new set of factors which are apart from the effects of the treatment with different concentrations of nutrient solution.

The Girdling Experiment

The experiment with girdled plants failed to show any

effects of the treatment on the disease development.

This indicates one of two things: either the girdle was not sufficiently effective in preventing the downward flow of the assimilation products, or the susceptibility is not influenced by these products.

The type of girdling which was used probably did not completely prevent the passage of the assimilation products. Although an accumulation did result, as evidenced by the swelling of the stem immediately above the point of treatment, it is possible that the difference in concentration was not adequate to exert an influence on the growth of the fungus. Furthermore, it could be that the assimilates are rapidly converted into relatively inert products used in the formation of new cells (manifesting themselves in the swelling). If this was the case no response could be expected on the part of the fungus. Considering this it seems that a statement to the effect that the metabolites are without an effect on susceptibility should be withheld until further evidence has been collected.

On the Determination of the Relative Resistance of Hybrids

The question remains: Can treatment with different strength of nutrient solution be used as a tool in determining the relative resistance of hybrid chestnut at the seedling stage? The difference in resistance between the native seedlings and the Asiatic was clearly expressed throughout the experiment and increased as it progressed.

The plants all showed this regardless of the strength of the nutrient solution on which they were growing. Superficially it appears that the method could be used successfully. However, the variations are so large that a large number of plants and a definite statistical design would be necessary to prove anything. For example, 9.40 per cent of the American chestnut seedlings showed no sign of the disease at the end of the experiment. The probability is very remote that these plants really are immune to the fungus; rather, their apparent immunity must be considered the result of a failure of the inoculations. In other words, based on experience it is possible to eliminate faulty results. Had it been a group of hybrid seedlings, this would have been impossible, because the results could not be predicted quite accurately in advance and interpreted accordingly.

Consider on the other hand the inoculation of Chinese seedlings on the 1/9 concentration and 72-day treatment. On August 22 two plants were not diseased (class 1), 2 were in class 2, 8 in class 3, 3 in class 4 and none was dead (class 5). Knowing the resistance of the Chinese chestnut, the occurrence of 3 plants in class 4 would raise a doubt in the mind, and the plants would be held for further examination. Had the plants been hybrids, this precaution may not have been taken and three perhaps, valuable plants would be lost. It is possible that this predicament would have been avoided had the plants been held

longer. Although they appeared to be completely girdled the fungus may have been limited to the outer bark, effectively walled off from the underlying cambium.

Summarizing the situation, it seems that the technique cannot be used in its present form in a critical study of the resistance of individual hybrid seedlings. For the comparison of large groups of relatively homogeneous seedlings it may be quite effective, although small differences in resistance hardly can be expected to show up.

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