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A comparative study of phenolics in chestnut (Castanea), and their relationships with resistance to Endothia Parasitica

Paul Edward Barnett

To the Graduate Council:

I am submitting herewith a dissertation written by Paul Edward Barnett entitled "A comparative study of phenolics in chestnut (Castanea), and their relationships with resistance to Endothia Parasitica." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Plant, Soil and Environmental Sciences.

B. S. Pickett, Major Professor

We have read this dissertation and recommend its acceptance:

Eyvind Thor, James Hilty, Gordon Hunt

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December 1972

To the Graduate Council:

I am submitting herewith a dissertation written by Paul Edward Barnett entitled "A Comparative Study of Phenolics in Chestnut (Castanea), and Their Relationships with Resistance to Endothia parasitica." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Agricultural Plant and Soil Science.

Major Professor

We have read this dissertation
and recommend its acceptance:

Accepted for the Council:

Vice Chancellor for
Graduate Studies and Research

A COMPARATIVE STUDY OF PHENOLICS IN CHESTNUT (*CASTANEA*), AND THEIR RELATIONSHIPS WITH RESISTANCE TO *ENDOTHIA PARASITICA*

A Dissertation

Presented to

the Graduate Council of

The University of Tennessee

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy

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by

Paul Edward Barnett

December 1972

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ABSTRACT

American chestnut (Castanea dentata Borkh.) was a highly valued forest species in the Appalachian region prior to introduction of the chestnut blight fungus (Endothia parasitica (Murr.) A. & A.), which practically eliminated it. Since no control method has been devised and oriental species and hybrids are not generally satisfactory replacements, breeding resistant individuals through selection offers some hope of reestablishment. A chemical method for screening resistant individuals for retention in a breeding program would be of great benefit. Since phenolic compounds have been implicated in disease resistance of some cultivated crops and forest and fruit trees, this study is an initial attempt to develop a chemical screening method by studying comparative phenolic composition of five types of inner bark samples.

Inner bark of individual American chestnut trees was sampled from sound and infected stems, sound roots and debudded twigs. Sound inner bark from exotic (blight resistant) chestnut individual trees was also sampled. A stepwise extraction of air-dried bark samples was carried out in a Soxhlet apparatus using four organic solvents of increasing polarity; this resulted in twenty crude extract groups. Each extract group was investigated by one- or two-dimensional thin-layer chromatography. Phenolic compounds were located by spraying chromatograms with ferric chloride-potassium ferricyanide. Over 225 phenolic components were located. In some cases components from different samples extracted with the same solvent were indistinguishable.

Some components were tentatively placed into more specific chemical classes. These compounds were found to most likely be derivatives of flavonoids, catechins, leucoanthocyanins, guaiacol and vanillic acid.

A distinctive difference in chromatographic pattern was noted between American chestnut sound bark extracts and infected bark extracts from the same trees. There was almost no correlation between American chestnut root bark extracts and stem bark extracts. Little correlation was noted between American chestnut and exotic chestnut extracts; only eight components were found to be indistinguishable. Several similarities were noted between American chestnut sound stem bark and debudded twig extracts from the same trees. 1972

Individual tree data indicated considerable qualitative and quantitative variation among American chestnut trees in the extractable phenolic composition of their inner bark.

A method adaptable to small quantities of extract was applied to bioassays with E. parasitica. In general, bioassay results did not correspond with observed field resistance to the chestnut blight fungus. Extracts from American chestnut infected bark extracts were most inhibitory; those from root bark of the same species least inhibitory.

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INTRODUCTION

American chestnut (Castanea dentata Borkh.) was an extremely important member of the Appalachian forest community prior to introduction of the chestnut blight fungus, Endothia parasitica (Murr.) A. & A. This pathogen spread throughout the Appalachians during the 30 to 40 years following its introduction, practically eliminating American chestnut. This highly desirable species was replaced by an inferior mixed oak association (Woods, 1957).

Federal and state organizations attempted to develop means of controlling the blight, but no satisfactory method has been developed to date. The possibility of breeding trees with natural resistance to the fungus offered the greatest hope of returning American chestnut to its former position in the forest community. Since Asiatic chestnuts (C. mollissima Blume. and C. crenata Sieb. & Zucc.) exhibit a high degree of resistance to the blight, it appeared that interspecific hybridization would provide a satisfactory means of replacing American chestnut. This has not proven to be the case, however. Hybridization results indicate there is a genetic linkage between the undesirable growth form (from a forest tree standpoint) of the Asiatic chestnut and the high resistance they exhibit to the fungus. Hybrid trees which exhibit the desirable forest tree form of American chestnut have not possessed the required blight resistance.

Results from a study of canker areas of hybrod backcross (mollissima x dentata x mollissima) trees inoculated with the blight fungus suggested to Clapper (1952) that at least two pairs of genes

control resistance to the chestnut blight. This view is apparently shared by Jaynes (1969) who suggests that resistance to the chestnut blight fungus is controlled by two or more recessive genes. This suggestion of multiple gene control of blight resistance strengthens the hypothesis that it should be possible to breed blight resistant trees through selection of large, living individual American chestnut trees and using them in a breeding program.

As Jaynes and Graves (1963) pointed out, the primary hindrance to more rapid production of chestnut cultivars is the lack of an efficient test for screening seedlings for blight resistance. A period of 10-15 years would normally be required for each breeding cycle (selection, propagation, crossing, outplanting, and progeny testing). Progeny testing requires most of this time because juvenile American chestnut trees seem to possess some resistance to the blight (Nienstaedt, 1951). Artificial inoculation is not a dependable means of testing resistance; trees may resist repeated inoculations, only to be attacked later when they show little or no resistance (Nienstaedt, 1953). If a method could be developed to determine the eventual field of resistance of a progeny or selection, a breeding program would be greatly accelerated and great savings would be realized in time, labor, and growing space.

Results reported by Nienstaedt (1953) suggest that it should be possible to develop a chemical screening technique. He reported that *in vitro* growth of the blight fungus on media prepared from water-soluble extracts of inner bark from three species of chestnut was significantly correlated with known resistance of the species in nature. Barnett (1967) did not obtain similar results, although differences

were observed in growth rate of the fungus on media prepared from water-soluble extracts of inner bark from Chinese and American chestnut individuals. Inner bark has been used in all cases, since American chestnut can be infected by the blight fungus only if the outer bark is broken by some natural or mechanical means (Murrill, 1906; Metcalf and Collins, 1911; Anderson and Babcock, 1913; Shear, Stevens, and Tiller, 1917). There appears to be a difference in the way the inner-bark cellular constituents of Chinese and American chestnut trees are metabolized by the fungus.

This study has five objectives: (1) to determine whether there are differences in the extractable phenolics of inner bark from exotic and American chestnut trees which could account for observed differences in resistance; (2) to tentatively identify (from chromatographic behavior and color reactions) these phenolic components which are common to resistant and susceptible species; (3) to determine whether there is quantitative variation in some extractable phenolics of American chestnut which would suggest multigenic control of resistance; (4) to investigate reported differences in tannin content of American chestnut trunk and root bark for qualitative and/or quantitative differences; and (5) to determine whether inner bark components extracted with organic solvents inhibit E. parasitica in vitro.

CHAPTER I

REVIEW OF THE LITERATURE

I. Historical

There is little doubt that American chestnut was one of the most valuable timber species found in Appalachian forests (Anderson and Rankin, 1914). Uses and value of this species have been reviewed by The U.S. Census Bureau (1907), by Butterick et al. (1925), and by Clapper and Gravatt (1943).

Once the chestnut blight fungus was discovered and isolated in the United States (Merkel, 1906) and shown to be pathogenic to Castanea dentata (Murrill, 1906), several pathologists became involved in a discussion concerning its origin. A group led by Clinton (1913) felt the blight fungus was native to the United States, becoming pathogenic to American chestnut because of predisposing factors such as continued coppicing of stands, severe winters or drought. The second group (Metcalf, 1908; Anderson and Rankin, 1914) noted that the disease was spreading from areas in which Japanese chestnut (C. crenata) had been planted as early as 1876. The latter group was proven correct when E. parasitica was identified in Japan and China (Shear and Stevens, 1913; Shear, 1916) and described morphologically by Shear et al. (1917). During these studies it was noted that the American chestnut could only be infected by the blight fungus through wounds in the bark which penetrated the outer green cortex (Murrill, 1906; Metcalf and Collins, 1911; Anderson and Babcock, 1913; Shear et al., 1917).

Once pathologists obtained some knowledge of the characteristics of the fungus they began attempting to devise a method for controlling its spread. These studies produced considerable knowledge of its reproductive habits and mode of dissemination (Anderson and Rankin, 1914; Studhalter and Heald, 1915; Heald and Gardner, 1914; Heald and Studhalter, 1914; Anderson and Babcock, 1913; Heald and Walton, 1914; Heald et al., 1915).

There was considerable discussion concerning the mode of operation of the fungus on infected American chestnut trees. Shear et al. (1917) noted that only on C. dentata did the blight fungus form a mycelial fan beneath the bark. Some pathologists felt that this characteristic indicated that single hyphae could not penetrate living cells; only after a quantity of mycelium had accumulated in injured and dead cells did the fungus push through the living bark tissue by mechanical force (Anderson, 1914; Keefer, 1914). Rankin (1914), however, felt that "the host cells just in advance of the edges of the fan disintegrate and form a distinct genatinous band which can be seen with the naked eye," suggesting that the fungus effects host cells enzymatically. Another investigator (Bramble, 1936) felt that both processes were involved; the primary invasion of American chestnut bark was accomplished by mass action of the mycelial fan followed by penetration of individual hyphae into cells along the sides of the fan. This secondary invasion was "accompanied by certain microchemical changes in infected cells."

Hyphae penetrate to the innermost layer of sapwood soon after the mycelial fan has reached the cambial layer (Bramble, 1938). Penetration of the sapwood occurs through wood and pith rays, with ray

parenchyma being invaded later. No hyphae are found in vessels until the later stage when the tree crown is already partly dead. Conduction of water and nutrients through xylary tissue is not stopped by direct action of the fungus; apparently the fungus stimulates parenchyma adjacent to vessels, forming tyloses which cause actual stoppage of conduction.

Numerous detailed accounts of the symptoms and signs associated with the blight fungus on American chestnut trees have been published (Merkel, 1906; Metcalf and Collins, 1911; Metcalf, 1912; Keefer, 1914; Shear et al., 1917; Diller, 1965). Symptoms include dead limbs or "flags" in summer, or, if low enough to be seen, reddish swollen or sunken cankers. A closer look at these discolored areas usually reveals the signs of the disease; either long, curling yellow tendrils (spore horns) or reddish-brown pustules (perithecia).

II. Disease Resistance

Indications of Resistance

On young sprouts of American chestnut which show no resistance, the fungus grows rapidly beneath the smooth outer bark. The bark is not broken and may be raised only slightly. Spreading mycelium can be seen as an orange discoloration under the thin bark (Nienstaedt, 1951).

When older sprouts are attacked, the outer bark may appear to remain intact because it usually takes longer for the fungus to kill the sprout. Only when red perithecia can be seen pushing through the destroyed bark can the fungus be identified. 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 species and hybrids sunken cankers are surrounded by an irregular line of callus mixed with dead tissue. The newly formed callus is continuously reinfected by the blight, which continues to extend the canker (Nienstaedt, 1951).

Trees exhibiting the hypertrophied canker reaction prevent the blight fungus from penetrating toward the cambium by forming wound tissue. This type of canker is noted on individual trees of all major species of Castanea. Wound tissue will check the advancing fungus only temporarily in C. dentata, but often walls off the fungus completely in C. mollissima and C. crenata. Until more exact information has been obtained, the wound tissue wall is one of the criteria which can be used in selecting American chestnut parent trees for use in a breeding program (Nienstaedt, 1951).

Inheritance of Resistance

Our knowledge regarding the inheritance of blight resistance is rather limited at present because: (1) although a large number of hybrids have been produced, the number of individuals representing any one cross is relatively small; (2) parents of a species hybrid are heterozygous and segregation takes place in the F_1 generation; and (3) while pure-line breeding could be used, chestnuts are self-sterile, and pure lines can be obtained only by repeated sibbing, a much slower process than selfing (Nienstaedt, 1951).

Present knowledge suggests that disease resistance is the result of a multiple-factor inheritance rather than a one-gene difference

(Graves, 1950; Jaynes, 1969); pronounced variation in resistance within the wild species seems to indicate this. This variation pattern could, perhaps, be explained on the basis of a number of mutant alleles determining resistance, but it seems doubtful that this is the case since it would result in a limited number of disease-resistant classes in the hybrids. Breeding records of the Connecticut Agricultural Experiment Station seem to indicate that hybrids show a continuous gradation of resistance between the two parents (Nienstaedt, 1951). It is as yet impossible to arrive at an estimate of the number of factors involved.

Nienstaedt (1951) concludes from studies of resistance in Japanese and Chinese chestnut that more factors are involved in disease resistance of the Chinese than of the Japanese chestnut. He based this conclusion on breeding records which indicated to him that while in Japanese-American hybrids the majority of individuals approached the latter parent in resistance, the opposite was the case in Chinese-American hybrids. It has been pointed out by Graves (1950) that there is evidence that disease resistance is linked with other characters. In the Chinese-American and Japanese-American hybrids it appears that blight resistance of the Asiatic species is closely linked to their inferior growth habit. This has been found to be a serious obstacle to the development of a resistant timber tree because an F_1 hybrid comparable to the Asiatic parent in resistance is, in most cases, valueless as a timber tree.

Castanea henryi is a forest tree in Central China, but is neither blight resistant nor frost-hardy enough for wide distribution

in the United States (Clapper and Gravatt, 1946; Graves, 1950). Castanea mollissima and C. crenata are not forest tree species, nor is C. crenata highly blight resistant. Other drawbacks common to all of these species is their requirement for the best sites available in the forest in order to survive and a lack of the ability to reproduce themselves under forest conditions (Clapper and Miller, 1949; Diller, 1950; Gravatt et al., 1953; Diller et al., 1964). American chestnut, on the other hand, is not very exacting in its site requirements, but prefers a deep, fresh, loose, and moderately fertile soil. Its development seems to depend more on the aspect and physical condition of the soil than on its chemical composition. It is a deep-rooted species which can derive its nutrition from the lower layers of earth (Zon, 1904). American chestnut can maintain itself over a wide variety of site conditions and reproduce itself with a minimum of light (Ashe, 1912).

Many species hybrids have been made and evaluated for blight resistance and stem form (Clapper, 1952; 1954). Berry (1960) found that 93 percent of C. dentata inoculated with the fungus were infected compared to 29 percent of C. mollissima.

By selecting phenotypes of American chestnut which show some resistance to the blight fungus and propagating them in a breeding orchard the trees may be crossed. Through repeated matings a high degree of resistance could be obtained in an American chestnut tree without hybridizing. Such a program has been initiated by the University of Tennessee, Forestry Department (MacDonald et al., 1962; MacDonald and Thor, 1967).

Types of Resistance

Disease resistance is usually divided into two classes. One type, controlled by specific single genes, imparts a high order of resistance to specific races of pathogens. The second type of resistance is controlled by several genes, each adding to the total disease resistance of a plant. The latter, as previously discussed, is the type of resistance believed to operate in Castanea. Although quantitative resistance such as this is not generally as great as the single gene type, it is not as subject to breakdown by new pathogenic races (Walker, 1959).

Additive resistance similar to that suggested to operate in chestnut has been noted in Western white pine (Pinus monticola Dougl.) resistant to the blister rust organism (Cronartium ribicola Fischer) (U.S. Forest Service, 1960). Squillace and Fischer (1966) demonstrated that differences in chemical constitution are strongly inherited in slash pine, while Hanover and Furniss (1966) tentatively showed there is a different monoterpene content in Douglas fir trees resistant to the Douglas fir beetle. Hanover (1966) also demonstrated genetic control of monoterpene concentration in Western white pine.

Formation of a layer of cork around an infected area is a common observation in plants, as, for example, in apple scab and the corky scab disease of potato. This reaction was discussed earlier in the case of Castanea species infected by E. parasitica. How effective this cork formation is in preventing further spread of the fungus has 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 advances (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." Bramble (1936), however, concluded that wound periderm found in hypertrophied cankers on infected American chestnut trees separated infected tissue from underlying bark. He felt that formation of wound periderm was a significant reaction for consideration in connection with resistance to chestnut blight. White pine (Pinus strobus L) trees resistant to the blister rust organism (Cronartium ribicola Fischer) have the ability to differentiate cork cambium wherever an infection occurs, walling off the invader with layers of cork and stone cells (Struckmyer and Riker, 1951). Susceptible trees showed no tendency toward wound periderm formation, hence, were less resistant.

Physiological resistance can also be expressed in two ways. The first is a passive type of resistance; i.e., toxic or inhibitory compounds are present prior to infection. The second is a dynamic type of resistance in which the defense system is activated in response to infection.

Passive resistance. Toxic compounds are produced by many plants. Skinner (1955) lists 64 known antibiotics that were extracted from 60 species of plants representing several families. Topps and Wain (1957) found that of 1,915 species of angiosperms, 23 percent contained fungitoxic compounds.

Since phenols occur regularly at infection and wounding sites and because many phenols and their oxidation products (quinones) are highly fungitoxic, these compounds have been associated with defensive mechanisms. Farkas and Kiraly (1962) and Cruickshank and Perrin (1964) have reviewed this literature thoroughly. Both reviewers conclude that only a few well documented cases merit listing in connection with their role in resistance.

Phenolics are synthesized in plants via either the shikimic acid pathway (McCalla and Neish, 1959; Levy and Zucker, 1960), the acetate-malonate pathway (Bentley and Keil, 1961; Bu' Lock and Smalley, 1961) or modification of both pathways (Underhill et al., 1957). Evidence has appeared indicating production, speed of production, or liberation of phenolics by plant tissue after infection is important in disease resistance (Davis et al., 1953; Kuc' et al., 1956; Muller, 1958; Cruickshank and Perrin, 1960; Kiraly and Farkas, 1962). The latter approach, dependent upon a specific host-pathogen interaction, offers an element of specificity difficult to realize when considering only preformed phenolics. The accumulation of phenolics has been detected in infected tissue as well as in healthy tissue in advance of the microorganism.

Coupled with the increase in phenolic content around infection and wound sites, an increase in phenoloxidase and peroxidase, in or adjacent to infected tissues, has often been reported (Farkas et al., 1959; Maine and Kelman, 1960). When one considers the antibiotic activity reported for quinones (Farkas and Ledingham, 1959; Kaul and Shaw, 1960), the localization of these compounds around points of infection and,

finally, the universal occurrence of plant phenolics, it is evident why researchers have often implicated these compounds with disease resistance in plants.

Little attention has been paid to the possible importance of polyphenols (aromatic compounds found in plants, possessing one or more phenolic hydroxyl groups) in disease resistance of forest trees (Hillis, 1962), Investigations have shown, however, that a high polyphenol content or the presence of certain polyphenols is associated with disease resistance in a variety of cultivated crops. LeTourneau et al. (1957) demonstrated that the growth of Verticillium albo-atrum in various concentrations of several phenols and quinones depended on the arrangement of the different chemical groups in the ring system. Similarly, Christie (1965) showed that the effect of several phenolic compounds upon two species of Phytophthora in microculture was highly dependent upon the number and position of substituents in the ring system.

That a plant constituent is inhibitory to an organism does not imply that the compound necessarily prevents infection. Hubbes (1962a, 1962b, 1966) isolated pyrocatechol, a glycoside, and a second undetermined phenol from the bark of a poplar species parasitized by Hypoxylon pruinatum (Klotz.) Cke. Although these compounds were toxic to the fungus, invasion of the bark proceeded unchecked. Perhaps these compounds are destroyed along with the host cell in advance of the fungus.

Concentration of a resistance-promoting compound may be more important than was at first realized. Echandi and Fernandez (1962) found

that two coffee species and their hybrid, which are resistant to coffee canker caused by Ceratocystis fimbriata Ellis and Halet., had a higher concentration of chlorogenic acid than did a susceptible species. High concentrations (625 ppm) of chlorogenic acid inhibited the fungus but low concentrations (39-78 ppm) stimulated fungal growth.

Tannins have long been suspected of being resistance factors. Offord (1940) studied the host-parasite relationships of Ribes and Cronartium and found that the decomposition products of the tannin mass of a highly susceptible and moderately susceptible species of Ribes showed the latter to contain more catechol tannins, while the former contained primarily the gallotannin type. Nienstaedt (1951) compiled data on the concentration of tannins in the different species of Castanea and found that resistance could not be explained simply by higher concentrations of tannin in the resistant individuals. The ranges in tannin content overlapped considerably, which would indicate a similar overlapping in the relative resistance of the three species studied, a condition not found in nature. He pointed out that the data may not allow interpretation, since they were compiled from analyses of material from trees of different ages. Clarke et al. (1942), however, reported that no important changes take place in the concentration of bark tannins during the life of a tree.

Because species of Castanea have a high tannin content, it has been suggested that some chemical-physiological relationship might be responsible for the resistance of Asiatic species and lack of resistance in American chestnut. Cook and Wilson (1915) in an elaboration of earlier work by Cook and Taubenhaus (1911), studied the effect

of various tannin-containing extracts of the American chestnut on the growth and spore germination of Endothia parasitica. Tannins were added to a synthetic growth medium in concentrations of from 0.1 to 2.8 percent. The summary indicated that: (1) 0.8 percent or more tannin, regardless of type, retarded spore germination, but later, frequently caused abnormal stimulation of aerial mycelial growth; (2) Endothia parasitica was able to use as much as 2 percent tannin for 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 Endothia parasitica.

Nienstaedt (1953) carried out a study involving water, alcohol, and ether extracts of different portions of bark from Castanea dentata, C. crenata, and C. mollissima. Extracts were made up in potato dextrose-agar plates and assayed with mycelium of the fungus. The water extract and the water soluble portion of an alcohol extract retarded growth of the fungus. Extracts from the most resistant species, C. mollissima, was most toxic, followed by extracts of the somewhat less resistant C. crenata. Extracts of highly susceptible C. dentata had little or no effect on fungus growth. Alcohol- and ether-soluble extractives also retarded hyphal growth, but relative toxicity of these extracts showed no correlation with relative resistance of the three species. When tannin extracts of bark from the three Castanea species

were purified, all retarded growth of the fungus if present above a specific concentration. There was no difference between the effect of tannins from Japanese and American chestnut, but those from Chinese chestnut were much more toxic. Total concentration of tannins in bark of the three species was not correlated with relative resistance of the species. In water extracts of the bark, however, concentration of tannins was greater in extracts from the resistant species. A qualitative test indicated that the tannin from Chinese chestnut was a pyrogallol-type, whereas the other two species contained a mixture of catechol and pyrogallol tannin. These results suggested to Nienstaedt (1953) that relative resistance of three species, at least in part, is a result of both differential solubility and qualitative differences between the tannins.

Byrde (1963) feels there is evidence to support the concept of resistance due to the presence or appearance of compounds not necessarily directly fungitoxic, but which have the ability to inactivate the extra-cellular enzymes of the fungus attempting to invade the plant. Among the extra-cellular enzymes involved, the pectolytic enzymes have assumed particular importance. Attention has been directed to them largely because of the fact that many fungi penetrate via the middle lamella, which is known to be predominantly pectic in nature.

Bazzigher (1955) studied enzymatic systems of E. parasitica by two dimensional paper chromatography. He found that: (1) at least a part of the Endothia enzyme system is an esterase which can function over the temperature range of 6° to 60°C; (2) this esterase system is an adaptive one, the enzyme not being formed if the proper substrate

is not present; (3) esterase activity occurs *in vivo* in *C. sativa* trees infected by the fungus but is highly localized, occurring only in the region of the lesion; (4) chromatograms of tannin extracts from healthy and infected bark of *C. sativa* show considerable localized "decomposition" of tannins in infected bark; (5) crude tannin extracts from Chinese and European chestnuts were about eight times more toxic to *Rhizoctonia solani* than the same extracts which had been subjected to the enzyme system of *E. parasitica*; (6) the enzyme system of *E. parasitica* includes peroxidase, specifically a polyphenoloxidase of the laccase type (a constitutional type as opposed to the adaptive esterase type); (7) this polyphenoloxidase is set free by mechanical wounding or by infection of *C. sativa* by *E. parasitica*; (8) enzyme systems other than esterase and laccase take part in the decomposition of the whole tannin complex.

Active resistance. Evidence for a dynamic state of resistance in plants is increasing rapidly. Many workers are now finding that chemical compounds responsible for resistant reactions are not present in the plant prior to infection, but are only produced after infection and interaction of the host and parasite. Compounds produced by plants in response to infection by a parasite and which impart resistance to that parasite are called phytoalexins.

The basic postulates of the phytoalexin theory were summarized by Muller and Borger (1940) and translated by Cruickshank and Perrin (1964) as follows:

> 1. A principle, designated as "phytoalexin" which inhibits the development of the fungus in hypersensitive tissue, is formed or activated only when the host cells come into contact with the parasite.

2. The defense reaction occurs only in living cells.
3. The inhibitory material is a chemical substance and may be regarded as the product of necrobiosis of the host cell.
4. This phytoalexin is non-specific in its toxicity towards fungi; however, fungal species may be differentially sensitive to it.
5. This basic response which occurs in resistant and susceptible hosts is similar. The basis of differentiation between resistant and susceptible hosts is the speed of formation of the phytoalexin.
6. The defense reaction is confined to the tissue colonized by the fungus and its immediate neighborhood.
7. The resistant state is not inherited. It is developed after the fungus has attempted infection. The sensitivity of the host cell which determines the speed of the host reaction is specific and genotypically determined (Cruickshank and Perrin, 1964, p. 528).

Cruickshank (1963a, 1963b) and Cruickshank and Perrin (1964) show cause for considering modification of postulates 4 and 5. That the selective toxicity of pisatin (a phytoalexin produced in peas) is well defined was demonstrated in a test that showed non-pathogens to be very sensitive to pisatin while pathogens were not (Cruickshank, 1962). Concerning postulate 5, Cruickshank and Perrin (1963) found the rate of increase in pisatin concentration is not a good measure of the resistance of susceptibility of a host.

Some of the other plants from which phytoalexins have been isolated and identified are sweet potato roots (Akazawa, 1960), orchid tubers (Gaumann et al., 1950) and carrot root tissue (Condon and Kuc, 1960; 1962).

Other types of compounds appear very similar to the phytoalexins but have not been included within its definition. These compounds are derived from chemical modifications of an existing molecule in the preinfectional state. Thus, oxidation of a non-toxic compound often produces molecules that are highly toxic. Lyr (1965) has reviewed the

literature on the toxicity of oxidized polyphenols. He concluded that nontoxic polyphenols are oxidized to toxic compounds but that afterwards a detoxification occurs by polymerization. The aglycone, the hydrolysis product of glycosides, is also often very toxic to microorganisms.

Hydroquinone, the aglycone of arbutin, is produced in pear leaves in response to Erwinia amylovora (Burr.) Winslow (Hildebrand and Schroth, 1964). They found that hydroquinone was inhibitory to the fungus and that B-glucosidose activity was low in susceptible tissues.

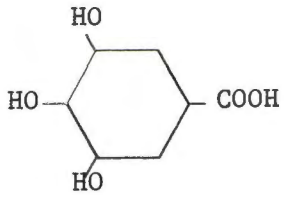
III. Phenol and Tannin Chemistry

Previous discussion has pointed out that the term "tannin" in older literature can only be relied upon to imply the presence of phenolic hydroxyl groups. It is now generally agreed that any naturally occurring compound of molecular weight between 500 and 3,000, containing one to two phenolic hydroxyl groups per 100 molecular weight, can be regarded as a tannin (Swain, 1965).

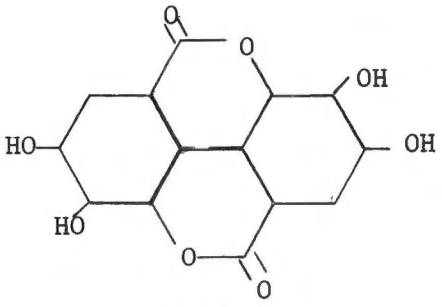
Freudenberg (1920) devised the vegetable tannin classification which is in use today. This system divides tannin into two main groups, mainly those which can be hydrolyzed by acids (hydrolyzable) and those which cannot be hydrolyzed by acids (condensed).

Hydrolyzable Tannins

Hydrolyzable tannins, as a class, are a combination of a polyhydric alcohol (such as glucose), the hydroxyl groups of which are partially or completely esterified by gallic acid (I) to form a gallotannin, or by ellagic acid (II), to form an ellagitannin.



I

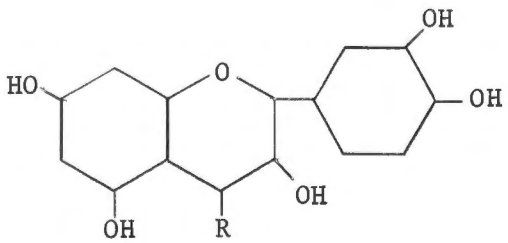


II

These tannins, upon hydrolysis with acids or enzymes, yield the carbohydrate plus the acid residue (Swain, 1965).

Condensed Tannins

Tannins of this class contain only phenolic nuclei. Although knowledge about this class is limited, it has been shown that they are generally formed by the condensation of two or more molecules of flavan-3-ols such as catechin (III) or flavan-3, 4-diols such as leucocyanidin (IV) or mixtures of the two. The hydroxylation patterns of the monomers vary, depending upon the source, but are generally related to the commonly occurring flavonols and anthocyanins. Attempts to hydrolyze these tannins to lower molecular weight substances yield instead a polymer which is often red or yellow in color, insoluble, and amorphous (Swain, 1965).



III - R = H

IV - R = OH

Structures III and IV fall into the general class of compounds called flavonoids. There are seven groups of compounds which fall into this class. They differ only in their oxidation levels, which is varied by the substituents on the center ring. Four other groups are related, in that they possess the C_6-C_3-C_6 basic structure. The eleven groups are: catechins, leucoanthocyanidins, flavonones, flavononols, flavones, anthocyanidins, flavonols, chalcones, dihydrochalcones, aurones, and isoflavones. The leucoanthocyanidins and catechins are colorless, the flavones and flavonols are widely distributed yellow pigments and anthocyanins are the common red to blue pigments of flower petals (Robinson, 1967).

Occurrence

According to White (1958) and Halsam (1966), Castanea dentata and C. sativa have hydrolyzable tannins present in their bark. Swain (1965) found these species to possess this same class of tannin in bark and wood. According to Hathway (1962), C. sativa contains 8 to 14 percent condensed tannin in its bark, while C. mollissima has 7 to 10 percent of an unspecified type of tannin in its wood (Hillis, 1962). Thus, there appears to be some uncertainty about the class of polyphenols to be found in Castanea.

White (1958) has discussed extensively the chemistry of both tannin classes, and King and White (1957) offer a plausible explanation for the apparent inconsistency concerning tannin types found in different parts of a plant. In studying the polyphenol content of leaves, young twigs, bark, sapwood, and heartwood of three species of Schinopsis (quebracho), they found the leaves produced a variety of compounds

similar to or identical with many already known components of typical hydrolyzable tannins. No condensed tannins or C_{15} components were present in leaves despite their known predominance in the heartwood. The only other identifiable polyphenols found in the leaves were quinic acid derivatives. Bark was found to serve as a storage center for these quinic acid derivatives and, in addition to traces of hydrolyzable tannin components, also contained traces of d-catechin. Sapwood showed a significant content of gallic acid, the gallotannin and two other hydrolyzable galloyl derivatives derived from the leaves, and was found to contain two primary and several secondary flavonoid components. The flavonoid components increased quantitatively as the sapwood-heartwood boundary was approached, and at that boundary hydrolyzable components suddenly disappeared while, a little way inside it, the d-catechin and two primary flavonoids also disappeared. In heartwood proper the unidentified condensed tannin molecules were accompanied by numerous secondary flavonols described above, and by traces of other materials found in the sapwood.

The overall picture seems to be one of synthesis of hydrolyzable tannin components in the leaves, transport of these substances to the sapwood with flavonoid compounds arising en route, possibly in the cambial layer. At the sapwood-heartwood boundary all of these substances participate in formation of typical heartwood condensed tannins of the quebracho tree. Here, indeed, lies the reason for the complexity of the condensed tannins of quebracho extract, and possibly the reason for inconsistencies in the literature concerning chestnut tannins.

Detection

Ferric chloride-potassium ferricyanide, with few exceptions, turns blue upon contact with phenols and tannins, detecting less than 1 μg of many of them (Hathway, 1969). Although not as sensitive as ferric chloride-potassium ferricyanide, the ferric chloride reagent turns hydroxyamic acids red, catecholic phenols green and pyrogallol phenols blue (Hathway, 1969; Hergert, 1960). Since catecholic phenols represent condensed tannins and pyrogallol phenols have been associated with hydrolyzable tannins, some impression of the comparative tannin makeup of an extract may be obtained with this reagent. The test is not completely reliable, however, so some care must be taken in using it. Deep blue-black color is evidence for presence of a 3, 4, 5-trihydroxy phenol (e.g., gallocatechin), but the formation of a green color does not necessarily indicate the absence of this group nor the presence of a catechol (o-dihydroxy group) (Robinson, 1967).

Simple phenolic compounds couple with diazonium salts in alkaline solutions to form azo dyes. Diazotized 4-nitroanaline, diazotized sulfanilic acid (Pauly reagent) and tetrazotized benezidine yield blue, purple, red and yellow spots. Thus, a phenol can be identified by its location and the color of its corresponding azo dye. Diazotized 4-nitroanaline yields rose-pink color with sinapic and ferulic acids, but is yellow with umbelliferone and scopoletin. Phenols of the phloroglucinol-resorcinol type yield red dyes with tetrazotized benezidine, while those of the pyrogallol-catechol type give ill-defined colors (Hathway, 1969; Roux and Maihs, 1960). In a study of tannins and polyphenols from conifer wood and bark Hegert (1960) found Pauly reagent

the most generally useful reagent because it yields different colors with a wide variety of phenolic compounds.

The use of ultraviolet light (UV), in the absence and presence of ammonia vapor, yields information useful in characterizing some phenolic compounds. In some cases the spots are revealed only by this means. Geissman (1955) found that in long-wave (360 nm) UV light some phenolic substances fluoresce (flavonols, chalcones) while others absorb and appear as dark spots against the fluorescence of the paper (flavonol glycosides, anthocyanins, flavones). When exposed to ammonia vapor while examined in UV light, flavones and flavonol glycosides fluoresce yellow, flavonones appear pale yellow and catechine pale blue. In white light, in the absence of ammonia vapor, flavonones appear yellow, anthocyanins blue-gray, chalcones and aurones orange-red.

CHAPTER II

MATERIALS AND METHODS

I. Plant Material

Plant material utilized, with data of collection, location, approximate elevation and diameter at ground level is presented in Table 1. All American chestnut trees were identified by the author. Exotic chestnut material was identified from TVA field sheets and records of the Bennet Hill progeny test, where collections were made. Trees 20 and 22 are full sibs from C. mollissima crosses while number 21 is a C. mollissima X C. alabamensis Ashe hybrid. All exotic chestnut material was originally introduced through the Plant Introduction Gardens, Savannah, Georgia, in 1925.

Stem or large limb sections of individual chestnut trees were collected and the cut ends covered with plastic bags to prevent drying. Within 24 hours of collection inner bark was removed to the xylem, after first removing the outer bark until no brown or green color was observable. Inner bark was cut into strips approximately 5 mm wide and allowed to dry approximately one week at room temperature (68-75°F) in paper bags, then ground in a Wiley mill to pass a 20-mesh screen.

II. Extraction

Samples of ground air dried bark (Table 2) were weighed, put into cellulose thimbles, and extracted in stages in a macro Soxhlet apparatus using certified American Chemical Society (ACS) reagents (Egger,

Table 1. Chestnut material collection areas, dates, elevations and sizes

No.	Diam. (in) Ground level	Date collected	Collection area	Approximate elevation (ft.)
American				
6	4	16 Oct 1971	Blue Ridge Parkway (Haywood Co., NC)	4200
7	6	16 Oct 1971	do.	4500
8	3.5	do.	do.	4800
9	6	25 Jan 1972	TVA Norris Reservation (Anderson Co., TN)	1000
10	7	11 Feb 1972	UT Forestry Woodlot (Knox Co., TN)	800
11	3.5	1 Mar 1972	Stratton Meadows-Tellico WL Mgt. Area (Cherokee Co., NC)	4000
12	5	14 Mar 1972	Albert Mtn., Coweeta Hydrologic Laboratory (Macon Co., NC)	4000
C. mollissima				
20	4	12 Jan 1972	TVA Norris Reservation (Anderson Co., TN) Bennett Hill Progeny Test, Block 5	1000
22	4	do.	do.	do.
Hybrid				
21	4	do.	do.	do.

Table 2. Trees sampled with size of air dried bark sample extracted and size of sample from each tree in each solvent applied to thin-layer plates for chromatographic analysis

Tree number	Sampling group	Weight of bark samples in grams	Size of sample chromatogrammed (in microliters) from:			
			Chloroform	Diethyl Ether	Acetone	Methanol
Am-6	Sound	10.0	4	2	6	5
	Infected	13.0	4	2	4	5
Am-7	Sound	10.0	4	2	6	5
	Infected	10.0	4	2	10	5
	Twigs	10.0	4	2	15	5
Am-8	Sound	10.6	6	2	5	5
	Infected	5.7	6	4	10	5
Am-9	Sound	13.0	4	-	7.5	4
	Root	10.0	4	-	10	5
	Twig	12.4	4	-	12	5
Am-10	Sound	10.0	4	-	6	5
	Root	10.0	4	-	10	5
	Twig	10.0	4	-	-	5
Am-11	Sound	10.0	6	-	6	5
	Twig	10.0	4	-	15	5
Am-12	Sound	10.0	4	-	6	5
	Root	10.0	6	-	10	5
	Twig	8.0	4	-	12	5
mollissima-20	Sound	10.0	8	-	15	5
C. hybrid-21	Sound	10.0	8	-	15	5
mollissima-22	Sound	10.0	8	-	15	5

1969). Solvents were used in the following sequence: petroleum ether (bp 40-60°C), chloroform, diethyl ether, acetone and methanol. In each case samples were extracted overnight (4:00 PM to 8:00 AM) or approximately 16 hours; preliminary work indicated that for some solvents this period was necessary to remove all soluble material. All extracts were concentrated to a volume of approximately 7 ml in a rotary evaporator and stored in a refrigerator (36-38°F) in cork-stoppered vials.

III. Thin-Layer Chromatography

Techniques for paper (PC) and thin-layer (TLC) chromatographic analysis of phenolic compounds have been discussed extensively (Roux, 1951; 1957; Geissman, 1955; 1962; Roux and Evelyn, 1958; Ibrahim and Towers, 1960; Bobbitt, 1964; Randerath, 1966; Egger, 1969; Hathway, 1969). By following these techniques, one- or two-dimensional chromatography was carried out on all except the petroleum ether fractions using 20 X 20 cm Eastman "Chromagram" type 6061 silica gel prepared sheets without activation. The petroleum ether fraction was not investigated chromatographically because only lipoidal materials would be included in it (Egger, 1969). Since some earlier bark samples were not the same size, an attempt was made to keep the size of chromatogrammed samples proportional to the weight of the bark sample; thus semi-quantitative data could be obtained. Samples were applied with disposable micropipettes of appropriate capacity.

Chloroform- and ether-soluble fractions were satisfactorily resolved by one-dimensional chromatography. It was necessary, however, to develop methanol- and acetone-soluble fractions in two directions

to obtain satisfactory component spot resolution. Solvent systems which proved most satisfactory were:

Chloroform fractions:
Benzene-methanol (90:10)

Diethyl ether fractions:
Benzene-acetic acid (85-15)

Acetone fractions:
1st. Ethyl acetate-methanol-water (100:16.5:13.5)
2nd. n-Butanol-acetic acid-water (63:10:27)

Methanol fractions:
1st. 2% acetic acid
2nd. n-Butanol-acetic acid-water (63:10:27)

All solvent components were certified ACS-grade chemicals, except glacial acetic acid, which was reagent grade. Distilled water was used in all cases. Solvents for the chloroform and ether fractions were used twice; those for the acetone and methanol fractions only once to avoid changes in solvent composition due to evaporation.

One-dimensional chromatograms were developed in the Eastman Chromagram developing apparatus (#6071). In order to facilitate production of the large number of required two-dimensional chromatograms the Camag multi-sandwich unit, designed for preparative-layer chromatography was adapted. To use this method, a strip of the layer 10 mm wide was removed from all four sides of the plate. Samples were applied 1.5 cm from the edge of the layer and dried with a hair dryer, if necessary (depending on the solvent). A few drops of water was distributed over a regular 20 X 20 cm glass chromatographic plate and the spotted precoated sheet laid in it, followed by a cardboard spacer 2.3 mm thick (Camag #25-223). This procedure was repeated until a total of four chromatographic plates and spacers and five glass

plates had been stacked to make a "sandwich." The sandwich was clamped together with two stainless steel bands, each with two set screws (Figure 1). To develop the chromatograms it was necessary to use chromatorgraphy tanks without a ridge in the bottom, using 150 ml of solvent per tank. All plates were developed at room temperature (65-75°F) after 30 minutes tank saturation. They were developed 12 cm instead of the standard 10 cm to obtain maximum resolution of component spots. Migration distances of component spots on chromatograms are expressed as hR_F-values, where

$$hR_F = \frac{\text{distance of spot center from starting point}}{\text{distance of solvent front from starting point}} \times 100$$

Resulting chromatograms were treated with a number of phenol detection reagents prepared according to the data in Table 3. Reagents were applied as sprays, using commercially available aerosol-type kits. Ferric chloride-potassium ferricyanide was used to detect phenol and tannin spots. Ferric chloride reagent was used to differentiate, if possible, the hydroxyamic acids, catecholic phenols and pyrogallol phenols. Diazotized 4-nitroanaline, diazotized sulfanilic acid (Pauly reagent) and tetrazotized benzidine were used in an attempt to differentiate further. Ultra-violet light with ammonia vapor was used to attempt the elucidation of flavones, chalcones, flavone glycosides, anthocyanins and flavones.

All of a specific sample type, i.e., sound stem bark, make up a sampling group; there is a total of five (Table 2, p. 27). A total of four solubility groups, made up of all samples extracted with one solvent are included. Therefore, the American chestnut sound stem

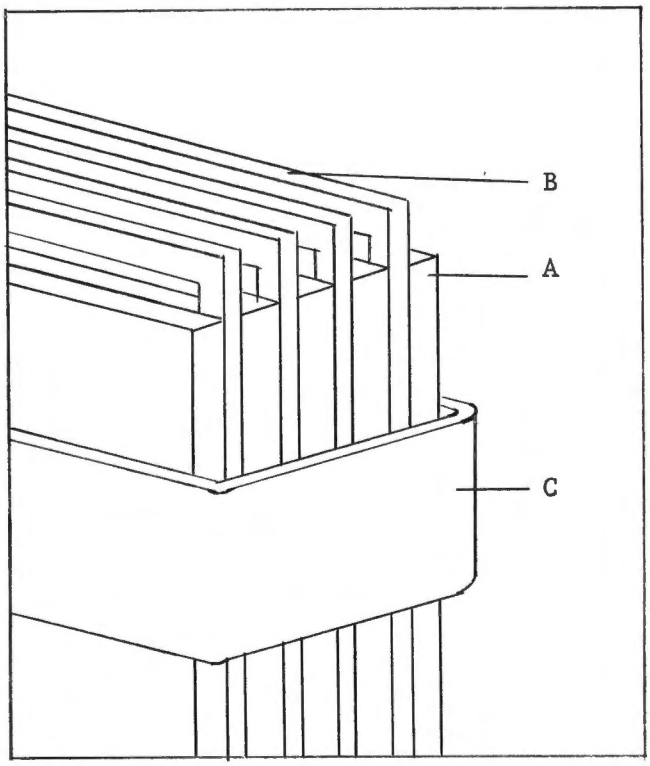


Figure 1. "Sandwich" arrangement used to develop four thin-layer chromatograms simultaneously. A-Standard 20 x 20 cm glass chromatographic plate; B-Cardboard spacers; C-Stainless steel band.

Table 3. Chromophoric reagents used to determine color reactions of extractable phenolic compounds of American chestnut sound, infected and root inner bark and twigs and exotic chestnut inner bark (Krebs, Heusser and Wimmer, 1969)

Benzidine, diazotized: for phenols	
Stock benzidine solution:	5 g benzidine and 14 ml 36% hydrochloric acid are diluted to 1000 ml with water.
Nitrite solution:	10% solution of sodium nitrite in water. Prepared freshly before use.
Spray reagent:	20 ml of the benzidine solution are mixed with 20 ml of the nitrite solution at 0°C, stirring continuously.
Diphenylboric acid, β-aminoethyl ester (Neu's reagent for natural products)	
Spray reagent:	1% methanolic solution of the β-aminoethyl ester of diphenylboric acid.
Procedure:	The layer is sprayed with about 10 ml reagent and the fluorescence colors observed in long-wave UV light.
Ferric chloride: for phenols and hydroxamic acids	
Spray reagent:	1-5% solution of ferric chloride in 0.5N hydrochloric acid.
Ferricyanide (potassium)-ferric chloride: for compounds with reducing properties, phenols, amines, thiosulphates and isothiocyanates	
Solution a:	1% aqueous potassium ferricyanide solution.
Solution b:	2% aqueous ferric chloride.
Spray reagent:	Equal amounts of a and b are mixed just before use.
4-Nitroaniline, diazotized (buffered): for phenols:	
Spray reagent:	5 ml 0.5% 4-nitroaniline solution in 2N hydrochloric acid are mixed under cooling with 0.5 ml 5% aqueous sodium nitrite solution and 15 ml 20% aqueous sodium acetate solution are added.
Sulphanilamide, diazotized (Pauly reagent according to Kutacek): for phenols, amines and heterocyclic compounds which can couple	
Spray reagent I:	3 g sulphanilamide are dissolved in a mixture of 200 ml water, 6 ml 36% hydrochloric acid and 14 ml n-butanol; 0.3 g sodium nitrite is added to 20 ml of this solution before spraying.
Spray reagent II:	10% aqueous sodium carbonate solution.
Procedure:	The layer is sprayed with I, left 5-10 min. and then sprayed with II.

bark, extracted with methanol makes up one sampling/solubility group while the American infected stem bark, extracted with the same solvent, makes up another; there is a total of 20 sampling/solubility groups. Eight samples of the crude extract from one individual tree within each group were chromatogrammed and a single chromatogram treated with each of the chromophoric reagents. The reagent which yielded the best results in terms of color differences and sensitivity was applied to single chromatograms of samples from the remaining individuals within a sampling/solubility group to obtain qualitative and semi-quantitative data on variation among individuals.

Specific colors for acetone- and methanol-soluble fractions were determined by comparison with color chips in the Munsell Book of Color (1929). For some spots it was impossible to obtain a match; such spots were compared with pastel colors (Talens Co., Apeldoorn, Holland) painted on chromatographic plates like those being used for separations. Because of the small amount of variation in color among spots of the chloroform and ether fractions they were described as they appeared to the eye. A difficulty in determining specific colors was also encountered with some spots found on chromatograms or acetone- and methanol-soluble fractions; when no match could be obtained from either the Book of Color or the pastels they were described as they appeared to the eye. Descriptive color abbreviations are presented in Table 4. These abbreviations were finally applied to all spots for ease of recognition and identification, because subtle differences in color intensity probably only reflect differences in the concentration of a component.

Table 4. Abbreviations used in identifying colors observed on chromatograms after spraying with chromophoric reagents

abs = absorbs	Aq = Aqua	Or = Orange
bt = bright	B = Black	Pk = Pink
d = dark	Bl = Blue	R = Red
du = dull	Bn = Brown	W = White
f = faint	Gn = Green	Y = Yellow
fl = fluorescent	Go = Gold	
l = light	Gy = Gray	
v = very		

Color reactions for each component spot on the chromatograms were recorded and an attempt made to assign the spot to a general chemical class according to the literature, using color and the hR_F-values obtained in the different solvents. Available phenolic standards were co-chromatogrammed with samples to permit direct comparison of hR_F-values and color reactions.

Mean hR_F-values and their standard errors were determined for each component spot, using all of a given sample/solubility group. Using the procedure described by Dice and Leraas (1936), comparing means including plus and minue two standard errors of the means ($\pm 2S\bar{x}$) it was possible to determine those spots found in a given sampling group which could possibly be identical to a spot or spots found in another sampling group. By using color reactions as further evidence, it was possible to limit the number of possibilities even more. Thus, if the

range of hR_F-values covered by $\pm 2S\bar{x}$ for two spots in different sampling groups overlapped, and if the color reaction for both was the same in most tests, they were taken to be the same compound. This procedure is illustrated by comparing acetone-soluble fractions of sound American chestnut and exotic chestnut bark. Mean hR_F-values for the spots of these two sampling/solubility groups are presented in Tables 5 and 6, with intervals covered by $\pm 2S\bar{x}$.

If the intervals covered by $\pm 2S\bar{x}$ for spots of both samples in solvent 1 are checked for overlapping range spots 2, 3, 5, 8, 9, 11, 12, 13, 14, 15, 17, 18, 20, 22, 24, and 25 of American sound stem bark extracts correspond with a total of 42 spots found in the exotic bark extracts. If, however, the hR_F-ranges of these component spots in solvent 2 are compared, it is found that only five possible matches remain (American 3, 12, 13, 18 and 25 with exotic 2, 9 and 12, 11, 10 and 7, respectively).

From the color reactions of these spots with Pauly reagent, however (Appendix Tables 12 and 13), it is noted that spot 12, American, is a dull orange, while spot 12, exotic, is pink; this match is eliminated. Spot 9 of the exotic extract is, however, a possible match, as both this spot and spot 12 of the American extract are brown.

Spot 13 of the American sound stem bark extract is a rather bright orange, while number 11 of the exotic is light pink. Spots 25 and 7 of the American and exotic extracts are both dull orange-brown while 18 and 10 are a faint pink. Thus, they are retained as possibly being the same compounds, leaving a total of four compounds found in the American chestnut sound inner bark and exotic chestnut

Table 5. Mean hR_F-values and the interval included by ± two standard errors for phenolic component spots of American chestnut sound inner bark extracts soluble in acetone, chromatogrammed on silica gel thin layers

Spot No.	No. Samples	Solvent 1[a]		Solvent 2[a]	
		Mean hR_F	Interval	Mean hR_F	Interval
1	4	7.2	4.2-10.2	39.2	36.4-42.0
2	7	10.3	7.5-13.1	68.0	66.0-70.0
3	8	9.0	8.3- 9.7	7.8	7.3- 8.3
5	8	18.8	16.9-20.7	53.5	52.1-54.9
6	8	30.8	27.8-33.8	45.4	14.2-46.6
7	8	31.9	29.1-34.7	69.1	66.9-71.3
8	8	39.4	37.2-41.6	29.1	28.1-30.1
9	8	45.6	43.4-47.8	46.4	45.1-47.7
11	2	43.5	38.5-48.5	20.5	19.5-21.5
12	7	41.8	38.2-45.4	55.4	53.2-58.7
13	5	46.4	42.6-50.2	72.6	70.0-75.2
14	8	51.2	48.9-54.0	53.7	51.9-55.5
15	4	48.5	46.6-50.4	60.5	59.2-61.8
16	5	52.0	49.0-55.0	73.6	70.8-76.4
17	8	57.0	54.6-59.4	72.5	70.1-74.9
18	5	47.2	42.8-51.6	85.2	82.4-88.0
19	7	30.0	28.1-31.9	18.8	17.8-19.8
20	4	14.5	13.2-15.8	46.0	43.4-48.6
22	2	18.0	16.0-20.0	27.5	26.5-28.5
24	3	25.0	22.0-28.0	46.3	43.9-48.7
25	3	31.7	24.3-39.1	50.7	48.1-53.3

[a]1. Ethyl acetate-methanol-water (100:16:13.5)
2. n-Butanol-acetic acid-water (63:10:27)

Table 6. Mean hR_F-values and the interval included by ± two standard errors for phenolic component spots of exotic chestnut sound inner bark extracts soluble in acetone, chromatogrammed on silica gel thin layers

Spot No.	No. Samples	Solvent 1[a]		Solvent 2[a]	
		Mean hR_F	Interval	Mean hR_F	Interval
1	4	1.8	0.3-3.3	5.5	4.9-6.1
2	4	9.2	7.9-10.5	9.2	8.2-10.2
3	2	14.0	12.0-16.0	49.0	49.0
4	4	18.8	17.1-20.5	31.8	30.8-32.8
5	2	26.0	26.0	61.5	58.5-64.5
6	4	36.8	36.3-37.2	68.0	65.8-70.2
7	4	37.5	36.5-38.5	48.5	46.5-50.5
8	2	39.5	36.5-42.5	65.5	64.5-66.5
9	4	44.8	43.3-46.3	57.5	55.8-59.2
10	4	50.0	48.8-51.2	83.8	82.3-85.3
11	4	42.5	41.2-43.8	73.0	71.2-74.8
12	2	37.0	35.0-39.0	60.0	58.0-62.0
13	1	37	37	55	55
14	2	22.0	22	48	48
15	2	14.5	11.5-17.5	35.5	34.5-36.5
16	1	58	58	90	90

[a]1. Ethyl acetate-methanol-water (100:16.5:13.5.
2. n-Butanol-acetic acid-water (63:10:27)

sound stem bark extracts which could be identical as determined by two-dimensional thin-layer chromatography followed by treatment with chromorphoric reagents.

Semi-quantitative data were obtained by comparing the area of each spot with the area of a circular template (Desaga standard; Brinkmann Instruments, Inc., Cantiague Rd., Westbury, N.Y. Cat. #25 09 200-7). Spots were placed into one of four categories as follows: absent, up to 30 mm^2, 30 to 200 mm^2 and over 200 mm^2.

IV. Bioassays

Crude extracts were bioassayed with Endothia parasitica by a method similar to that described by Olsen (1971). Six equally spaced circles 1.2 cm in diameter were drawn on strips of Whatman 3 MM chromatographic paper 3 cm wide and 13 cm long. Fifty microliters (µl) of crude extract was applied inside three of the circled areas with a 100 µl Hamilton 710-LT microsyringe; 50 µl of the appropriate solvent was applied to the three remaining circles. Treatments were assigned to strips and circles completely at random. Each test included six types of samples; five crude extracts and control. The five crude extracts included an American chestnut sound stem bark extract and a corresponding sound root bark extract. A second American chestnut sound stem bark extract corresponded to an infected stem bark extract. The fifth crude extract tested was always from sound bark of a C. mollissima individual or the hybrid.

The paper strips were exposed to a germicidal lamp in a tissue culture hood for 30 minutes, then transferred with sterile forceps to 15 cm sterile, disposable plastic petri dishes. Thirty milliliters

of dilute potato dextrose agar (PDA) (Appendix, p. 133) was poured over the strips and swirled about, completely covering the bottom of the dish; this resulted in a layer approximately 1 mm deep over the paper strip. After pouring, strips were pressed down with a sterile spoon-shaped spatula from the center outward to expel air bubbles trapped beneath the strip and to yield a uniform layer of media over the entire strip. After the media hardened single pycnidiospores of E. parasitica germinated on identical medium were transferred to the center of all circles. These single spores were obtained as outlined in the Appendix, p. 133. Initial stock culture of the blight fungus was obtained from a mycelial fan growing beneath the bark of an infected American chestnut seedling located in the University of Tennessee-Atomic Energy Commission irradiated American chestnut seedling orchard (Anderson County, TN). Mycelium from the fan was transferred asceptically to water agar plates and then to dilute PDA (Appendix, p. 133); stock cultures were maintained at room temperature on the latter medium, with new cultures being initiated every 30 to 40 days. Germinating spores and a plug of medium approximately 1.5 mm in diameter were cut from the plates and transferred, face down, to the circle. After 40 to 60 hours at room temperature plates were evaluated; those colonies which had grown out of the circle were scored (+) while those contained within the circle were scored (-).

Numbers of plus and minus observations were arranged in contingency tables and subjected to the Chi-square test for statistical significance (Cochran and Cox, 1957). To compare results of any treatment with control or with any other treatment the appropriate

2 x 2 contingency table was prepared and subjected to the Chi-square analysis, correcting for continuity as described by Snedecor (1957) and Steel and Torrie (1960). Treatment differences at the 5-percent level of probability were taken to be different.

CHAPTER III

RESULTS

I. Thin-Layer Chromatography

Introduction

Separation patterns on silica gel layers resemble those in paper chromatography (PC) so that the rules of thumb developed by Bate-Smith and Westall (1950) for partitioning organic solvents generally apply:

$$hR_F\ (R\text{-}CH_3) > hR_F\ (R\text{-}H) > hR_F\ (R\text{-}OCH_3) > hR_F(R\text{-}O\text{-sugar}).$$

As alkyl groups are introduced the hR_F-value rises; polar substituents decrease it. Carboxyl groups have about the same effect as hydroxyl groups and methoxy or acetoxy are roughly equivalent in their effect on hR_F (Egger, 1969).

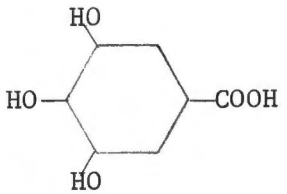
These effects are demonstrated to some extent in Table 7, which contains hR_F data for standards chromatographed in connection with the chestnut work. Gallic acid (I), with hydroxyl groups in the 3, 4 and 5 positions, consistently exhibits lower hR_F-values than salicylic acid (V), which has only one hydroxyl ortho to the carboxyl group. Similarly, pyrogallol (VI) consistently has slightly lower migration distances than catechol (VII). Phloroglucinol (VIII) demonstrates the effect of group position upon migration. It has the same number of OH-groups as pyrogallol, but all are meta, while in pyrogallol all are ortho.

Table 7. Mean hR_F-values of some phenolic compounds on silica gel thin layers in five solvent systems

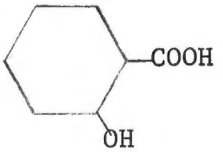
Compound	Solvent system[a] 1	2	3	4	5
	Mean hR_F-values				
Gallic Acid	0	0	14	72	64
Chlorogenic Acid	0	0	7	52	67
Salicylic Acid	8	65	29	95	96
Caffeic Acid	0	8	32	88	53
Fisetin	7	0	60	82	2
Myricetin	0	0	58	55	--
Rutin	0	0	21	60	47
Quercetin	Str[b]	0	--	85	2
Naringin	0	0	48	72	78
Apigenin	0	0	79	98	0
Catechol	30	35	84	98	87
Pyrogallol	22	7	81	84	85
Vanillin	--	--	98	98	78
Phloroglucinol	9	0	68	78	69
4-Methyl Catechol	36	33	86	98	83

[a]1. Benzene-methanol (90:10)
2. Benzene-acetic acid (85:15)
3. Ethyl acetate-methanol-water (100:16.5:13.5)
4. n-Butanol-acetic acid-water (63:10:27)
5. 2% acetic acid

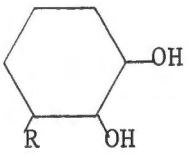
[b]Streak near origin.



I

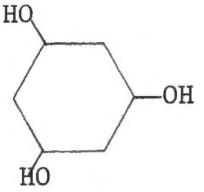


V



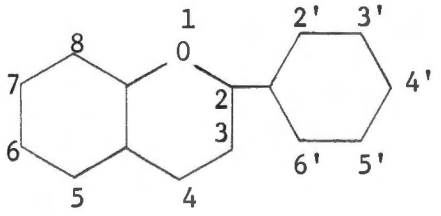
VI - R=OH

VII - R=H



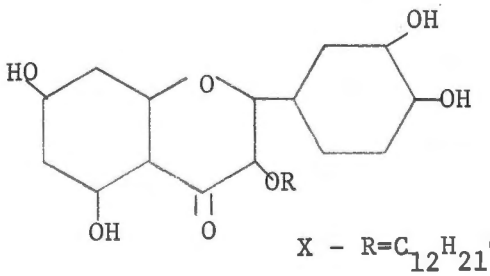
VIII

A notable characteristic of the paper chromatographic behavior of flavonoid compounds (IX) is the immobility of the aglycones of



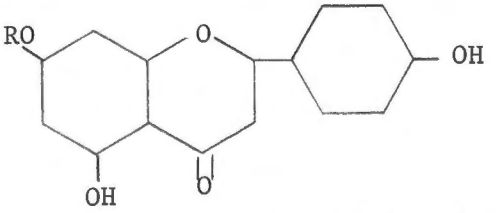
IX

flavonols, flavones and anthocyanidins, aurones and chalcones in aqueous solvents (Roux and Evelyn, 1958). This characteristic was also noted to hold true in the case of thin-layer chromatography on silica gel. While chromatographing standard compounds in conjunction with the chestnut work, it was noted (Table 7) that rutin (X) and naringin (XI) have hR_F's of 47 and 78, respectively, in 2% acetic acid while their corresponding aglycones, quercetin (XII) and apigenin (XIII), remain at or near the



X - R=$C_{12}H_{21}O_9$

XII - R=H



XI - R=$C_6H_{12}O_5$

XIII - R=H

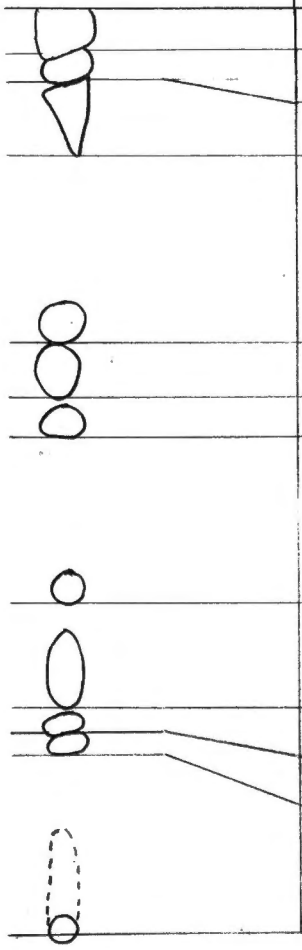
origin. In partitioning solvents, such as BAW, however, the aglycones migrate farther than their corresponding glycosides.

Chloroform-Soluble Fractions

No solvent among those recommended for chromatography of slightly polar phenolic derivatives provided satisfactory separation of components of these extracts. The one found satisfactory, benzene-methanol (90:10), is recommended by Stahl and Schorn (1969) for aglycones of anthraquinone derivatives.

Composite silica gel thin-layer chromatograms of material extracted with chloroform from American chestnut sound, infected and root inner bark and debudded twigs as well as exotic chestnut inner bark are presented in Figures 2 through 6, respectively. Mean hR_F-values and the colors produced with a variety of reagents are also presented in the figures. For ease of comparison a composite of all five one-dimensional chromatograms is presented in Figure 7. Table 8 contains a summary of component spots from the different sampling groups which could be identical compounds, as determined by comparing ranges of mean hR_F-values $\pm 2S\bar{x}$ and the color reactions for each.

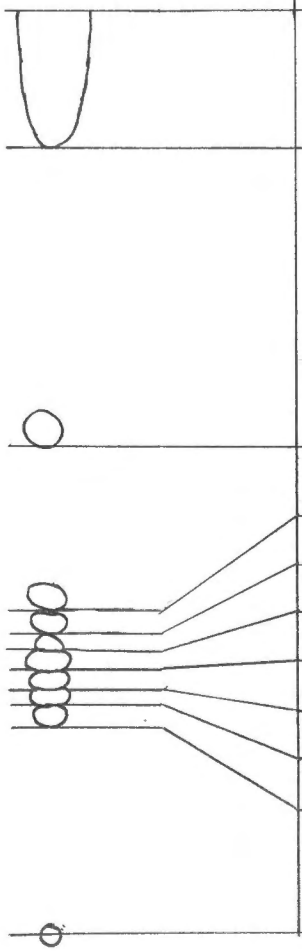
The facts that the components are soluble in chloroform and that they are chromatogrammed satisfactorily in a solvent as non-polar as benzene-methanol (90:10) suggest very little hydroxylation is present. The reaction of most spots with diazotized-4-nitroanaline, to yield faint brown or faint yellow colors, confirms this to a certain extent (Hathway, 1969). Most of the components of these extracts are probably substituted with alkyl or alkyl ether groups.



Spot No.	Mean hR_F	Colors with:						
		1	2	3	4	5	6	7
8	98	–	–	–	–	–	–	–
7	92	–	–	–	–	–	–	–
6	88	–	–	–	–	–	–	–
10	66	–	–	–	–	–	–	–
9	60	–	–	–	–	–	–	–
5	52	–	–	–	–	–	–	–
4	37	–	–	–	–	–	–	–
3	26	–	–	1-Bn	–	vf-Y	–	Y-fl
2	22	–	–	1-Bn	–	vf-Y	1-Bn	–
1	20	–	–	1-Bn	–	vf-Y	1-Bn	–

Figure 2. Composite thin-layer chromatogram of chloroform-soluble fractions of American chestnut inner bark. Chromatogrammed on Eastman Chromagram sheet, type 6061 silica gel with benzene-methanol (90:10) solvent; 12 cm migration. All spots positive with $FeCl_3K_3Fe(CN)_6$ reagent. Other reagents: (1) visible light, (2) 2% $FeCl_3$ in 0.5N HCl, (3) tetrazotized benzidine, (4) Pauly reagent I, (5) Pauly reagent I + II, (6) diazotized-4-nitroanaline, (7) Neu's reagent + 360 nm UV light.

Bn = Brown; Y = Yellow.
l = light; v = very; f = faint; fl = fluorescence.



Spot No.	Mean hR_F	Color with: 1	2	3	4	5	6	7
9	93	-	-	1-Bn	-	-	f-Bn	-
8	55	-	-	-	-	-	-	-
7	36	-	-	-	-	-	-	-
6	34	-	-	1-Bn	Y	f-yBn	1-Bn	-
5	32	-	-	1-Bn	Y	f-YBn	1-Bn	-
4	30	R	vf-Bn	d-R	bt-Y	Go	du-Y	-
3	28	f-Y	-	1-Bn	Y	f-YBn	1-Bn	-
2	26	f-Y	-	1-Bn	Y	f-YBn	1-Bn	-
1	24	f-Y	-	1-Bn	-	-	1-Bn	-

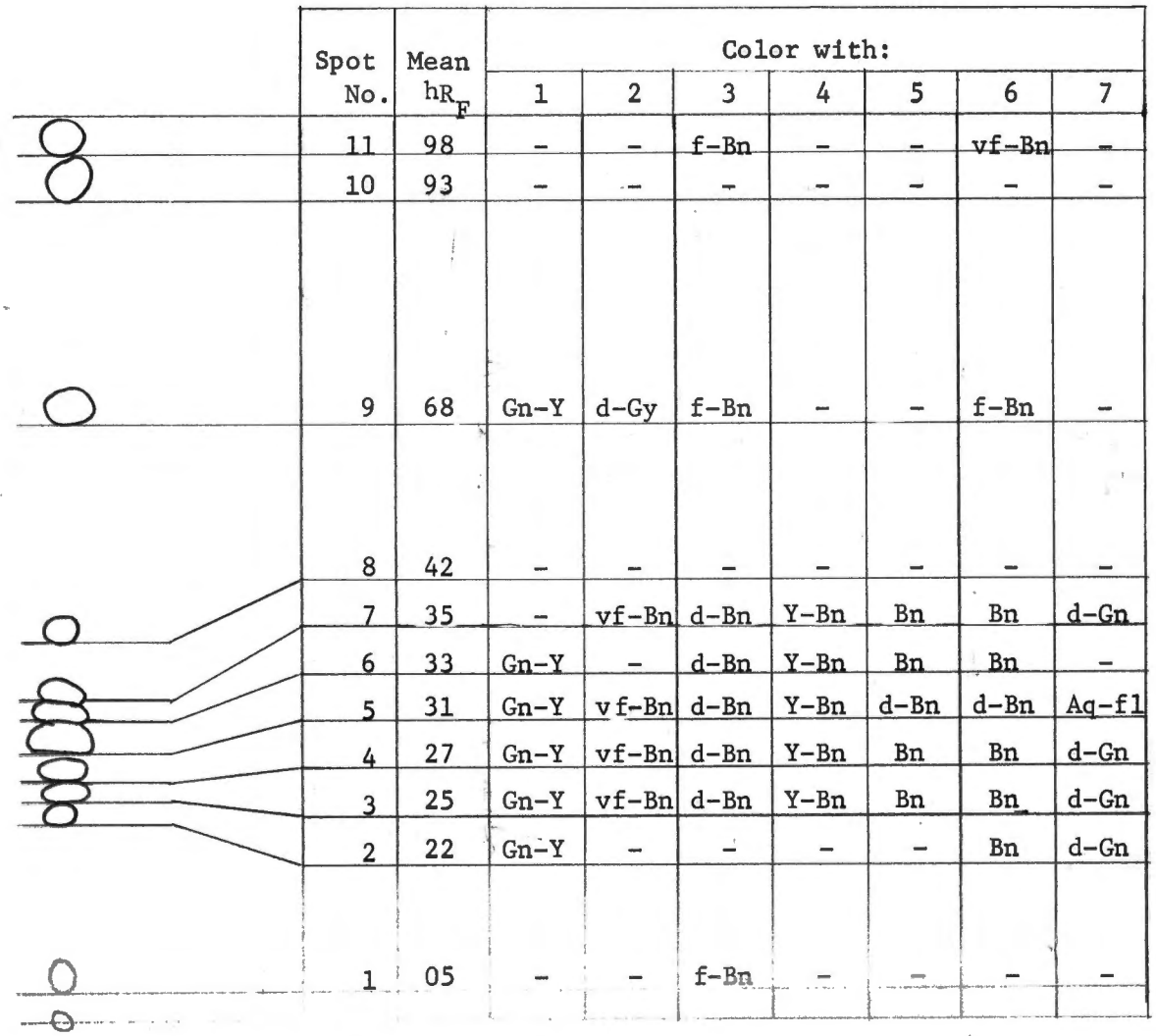
Figure 3. Composite thin-layer chromatograms of chloroform-soluble fractions of infected American chestnut inner bark. Chromatogrammed on Eastman Chromogram sheet, type 6061 silica gel with benzene-methanol (90:10); 12 cm migration. All spots positive with $FeCl_3$-$K_3Fe(CN)_6$ reagent. Other reagents: (1) visible light, (2) 2% $FeCl_3$ in 0.5N HCl, (3) tetrazotized benezidine, (4) Pauly reagent I, (5) Pauly reagent I + II, (6) diazotized-4-nitroanaline, (7) Neu's reagent + 360 nm UV light.

R = Red; Y = Yellow; Bn = Brown; Go = Gold.
v = very; f = faint; l = light; d = dark; du = dull; bt = bright.

Spot No.	Mean hR_F	Color with: 1	2	3	4	5	6	7
4	98	-	vf-Bn	l-Bn	vf-Y	vf-Y	-	RW-fl
3	36	-	-	vf-Bn	vf-Y	vf-Bn	-	-
2	27	f-Y	-	vf-Bn	vf-Y	vf-Bn	vf-Bn	-
1	23	f-Y	-	vf-Bn	vf-Y	vf-Bn	vf-Bn	-

Figure 4. Composite thin-layer chromatogram of chloroform-soluble fractions of inner bark from American chestnut roots. Chromatogrammed on Eastman Chromagram sheet, type 6061 silica gel with benzene-methanol (90:10) solvent; 12 cm migration. All spots positive with $FeCl_3$-$K_3Fe(CN)_6$ reagent. Other reagents: (1) visible light, (2) 2% $FeCl_3$ in 0.5N HCl, (3) tetrazotized benezidine, (4) Pauly reagent I, (5) Pauly reagent I + II, (6) diazotized - 4-nitroanaline, (7) Neu's reagent + 360 nm UV light.

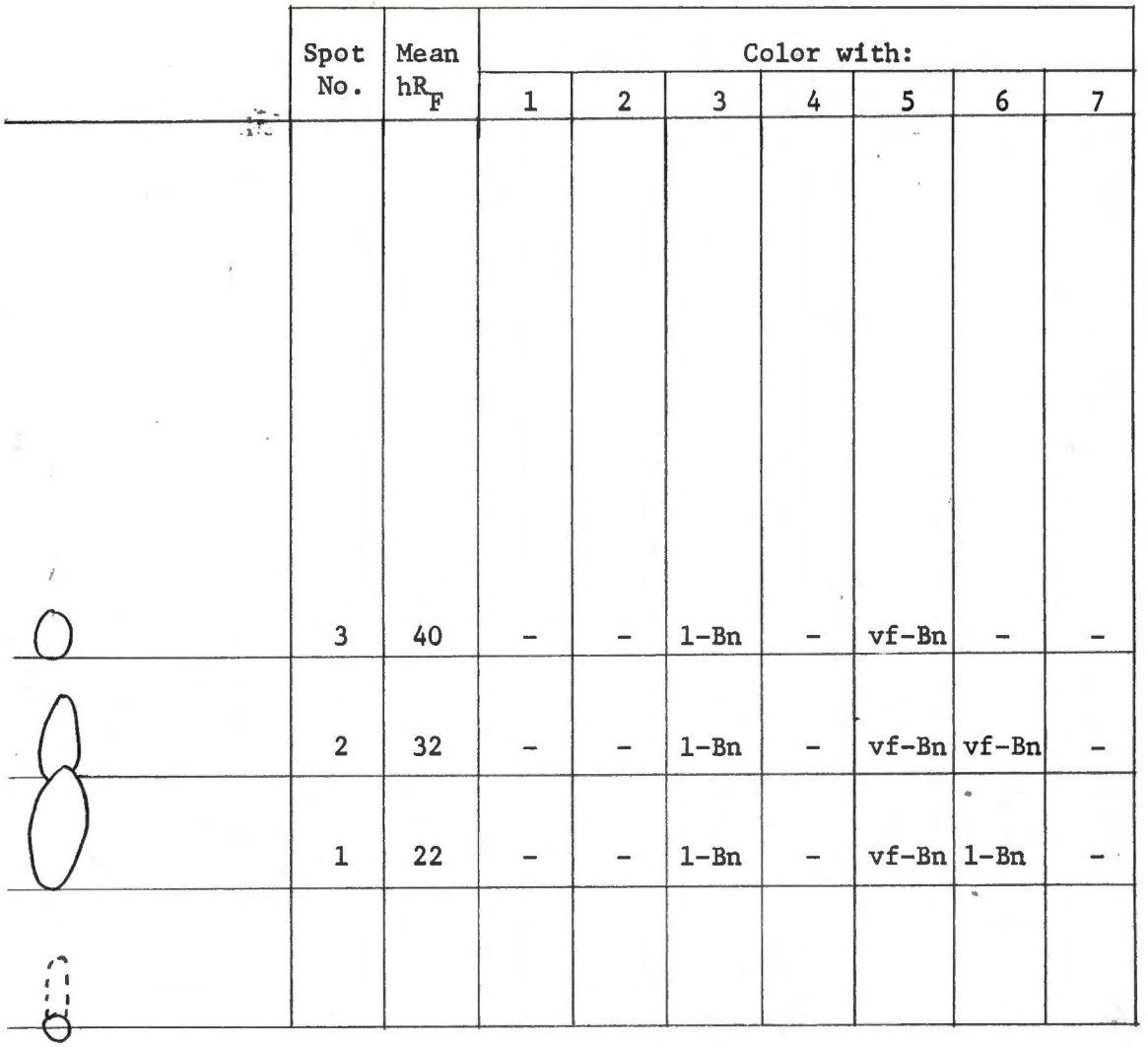
Y = Yellow; Bn = Brown; R = Red; W = White.
f = fluorescence; v = very; f = faint.



Spot No.	Mean hR_F	Color with: 1	2	3	4	5	6	7
11	98	-	-	f-Bn	-	-	vf-Bn	-
10	93	-	-	-	-	-	-	-
9	68	Gn-Y	d-Gy	f-Bn	-	-	f-Bn	-
8	42	-	-	-	-	-	-	-
7	35	-	vf-Bn	d-Bn	Y-Bn	Bn	Bn	d-Gn
6	33	Gn-Y	-	d-Bn	Y-Bn	Bn	Bn	-
5	31	Gn-Y	vf-Bn	d-Bn	Y-Bn	d-Bn	d-Bn	Aq-fl
4	27	Gn-Y	vf-Bn	d-Bn	Y-Bn	Bn	Bn	d-Gn
3	25	Gn-Y	vf-Bn	d-Bn	Y-Bn	Bn	Bn	d-Gn
2	22	Gn-Y	-	-	-	-	Bn	d-Gn
1	05	-	-	f-Bn	-	-	-	-

Figure 5. Composite thin-layer chromatogram of chloroform-soluble fractions of American chestnut debudded twigs. Chromatogrammed on Eastman Chromagram sheet, type 6061 silica gel with benzene-methanol (90:10) solvent; 12 cm migration. All spots positive with $FeCl_3$-$K_3Fe(CN)$ reagent. Other reagents: (1) visible light, (2) 2% $FeCl_3$ in 0.5N HCl, (3) tetrazotized benzidine, (4) Pauly reagent I, (5) Pauly reagent I + II, (6) diazotized 4-nitroanaline, (7) Neu's reagent + 360 nm UV light.

Gn = Green; Y = Yellow; Gy = Gray; Bn = Brown; Aq = Aqua.
v = very; f = faint; d = dark; fl = fluorescent.



Spot No.	Mean hR_F	Color with: 1	2	3	4	5	6	7
3	40	-	-	1-Bn	-	vf-Bn	-	-
2	32	-	-	1-Bn	-	vf-Bn	vf-Bn	-
1	22	-	-	1-Bn	-	vf-Bn	1-Bn	-

Figure 6. Composite thin-layer chromatogram of chloroform-soluble fractions of exotic chestnut inner bark. Chromatogrammed on Eastman Chromagram sheet, type 6061 silica gel with benzene-methanol (90:10) solvent; 12 cm migration. All spots positive with $FeCl_3$-$K_3Fe(CN)_6$ reagent. Other reagents: (1) visible light, (2) 2% $FeCl_3$ in 0.5N HCl, (3) tetrazotized benzidine, (4) Pauly reagent I, (5) Pauly reagent I + II, (6) diazotized-4-nitroanaline, (7) Neu's reagent + 360 nm UV light.

Bn = Brown.
v = very; f = faint; l = light.

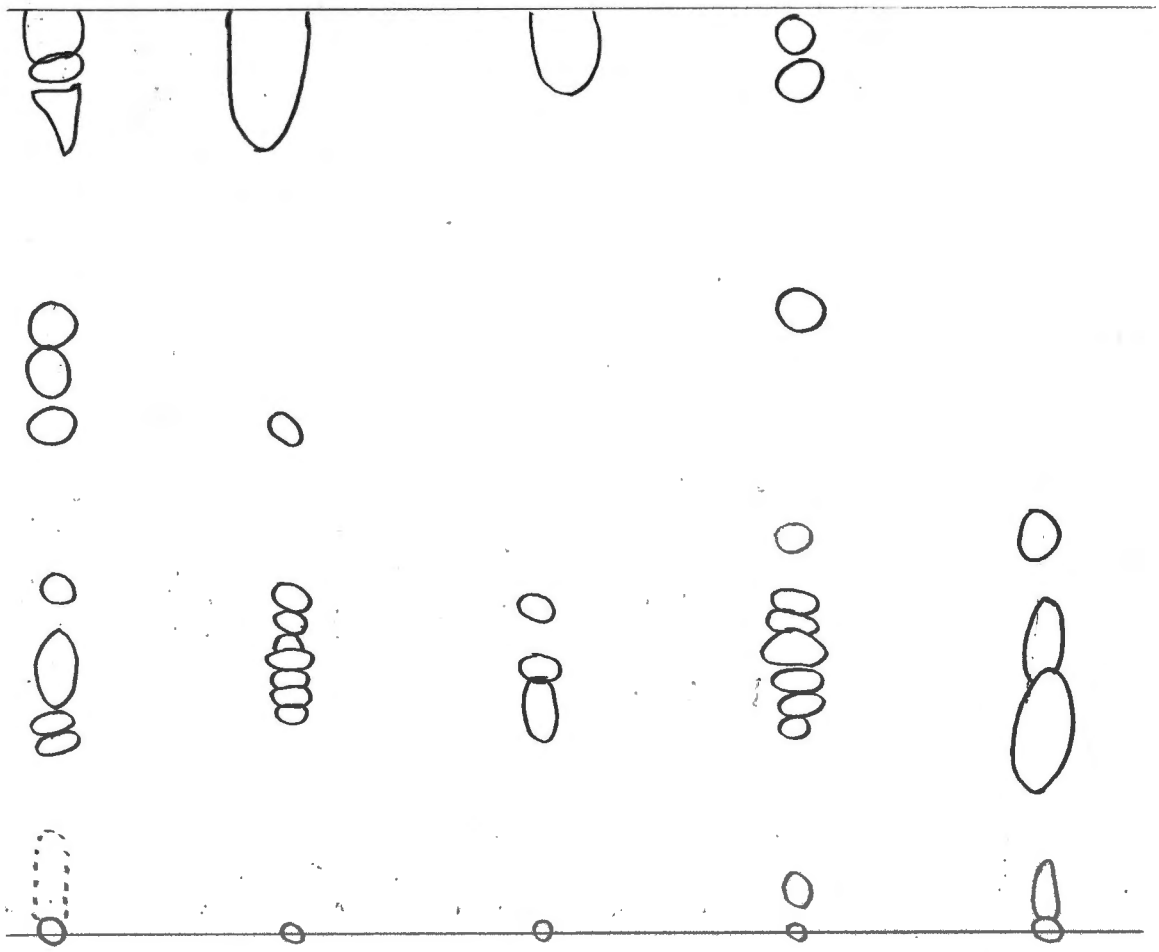


Figure 7. Composite thin-layer chromatogram: chloroform-soluble fractions of (from left to right) American chestnut sound, infected and root inner bark and twigs and inner bark of exotic chestnut. Chromatogrammed on Eastman Chromagram sheet, type 6061 silica gel with benzene-methanol (90:10) solvent; 12 cm migration. All spots positive with $FeCl_3$-$K_3Fe(CN)_6$ reagent.

Table 8. Spots which could represent the same compounds found in chloroform soluble extracts from American chestnut sound, infected, and root inner bark and debudded twigs and Chinese chestnut inner bark. Similarity established by comparing mean hR_F values with ±2 standard errors and color reactions

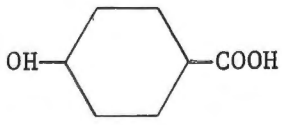
Sampling Group				
American Sound	American Infected	American Root	American Twig	Exotic
2	-	-	-	1
-	-	-	-	-
-	5	-	-	2
-	2	-	3	-
-	1	1	-	-

Only one spot found in the American sound bark extract corresponded with spots found in any of the remaining extracts. Spots 1, 2 and 5 of the American chestnut infected inner bark extract correspond with spot 2 of the exotic bark extract, spot 3 of the American twig extract and spot 1 of the American root bark extract, respectively. While several spots correspond when the hR_F-values and their ranges included in $\pm 2\bar{Sx}$ alone are compared, the color reactions do not correspond, excluding most of those which match.

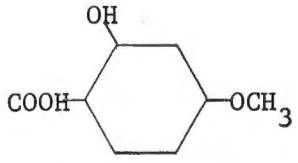
Because of the general lack of good, sharp, specific color reactions of the spots on chromatograms of this solubility group, it is difficult to classify many of them chemically. There are, however, some notable exceptions; spot 3 of the American sound inner bark extract (Figure 2, page 45) yielded a yellow fluorescence in 360 nm UV light after spraying with Neu's natural product reagent. This reaction

suggests a flavonoid compound (IX). The light brown color with tetrazotized benzidine also suggests a flavonoid of the 5, 7, 3', 4', 5' hydroxy type (Roux and Maihs, 1960) and the yellow color observed with Pauly reagent does not rule out this type of compound. In this case, however, the phenolic groups must be very highly methylated in order to observe the solubility reaction discussed earlier.

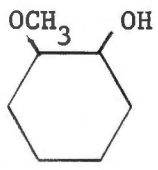
Another spot which yielded some characteristic reactions is number 4 of the American chestnut infected inner bark extract (Figure 3, p. 46). This spot did not react with Neu's reagent and is probably not a flavonoid. Its deep yellow color with diazotized-4-nitroanaline suggests a monohydroxy phenol (Hathway, 1969), and the reaction with Pauly reagent suggests a derivative of p-hydroxy benzoic acid (XIV), vanillic acid (XV), guaiacol (XVI) or guaiacol with an alpha-hydroxy group (Hergert, 1960).



XIV



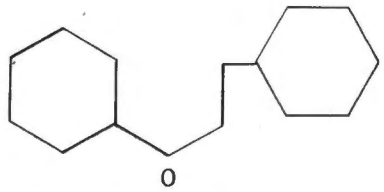
XV



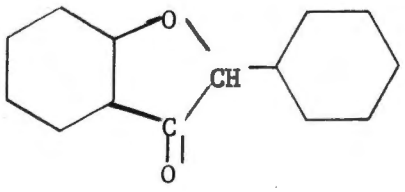
XVI

The red color obtained with tetrazotized benezidine suggests a phenol of the phloroglucinol-resorcinol type, or any compound possessing two meta hydroxyl groups. Whether one of these hydroxyls could be methylated and result in the same color reaction is not known; no evidence has been located in the literature. The faint brown color with ferric chloride suggests a 3- or 5-hydroxy flavone (Geissman, 1955) and in

spite of the evidence for another structure this class of compound cannot be completely eliminated simply because it does not react with Neu's reagent. Also, the fact that in visible light and in the presence of ammonia vapor a reddish color is obtained suggests a chalcone (XVII) or aurone (XVIII) (Geissman, 1955).



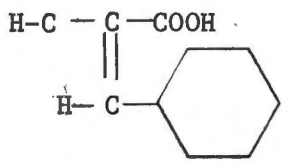
XVII



XVIII

Only spot 4 of the American chestnut root inner bark (Figure 4, p. 47) gave characteristically different color reactions. The faint brown color with ferric chloride suggests a 3- or 5-hydroxy flavone, as does the reddish-white fluorescence in 360 nm UV light after treatment with Neu's reagent. The color reactions observed with other reagents provide no evidence which conflicts with this possibility.

Extracts of debudded twigs of American chestnut trees were sampled in an attempt to correlate inner bark extract composition with the composition of twig extracts. Spots 2 through 4 and 7 of this extract (Figure 5, p. 48) yield essentially the same types of reactions with all reagents; spot 5 is similar, but fluoresces in 360 nm UV light after treatment with Neu's reagent and also in the presence of ammonia vapor. It is a dull green color in 360 nm light without any chemical treatment. These reactions suggest a derivative of O-coumaric acid (XIX) (Bate-Smith and Westall, 1950). All the spots exhibit the 3- or



XIX

5-hydroxy flavone reaction with ferric chloride and the complementing reactions with the remaining reagents.

Exotic chestnut inner bark extracts (Figure 6, p. 49) appear to have no components in common with American chestnut extracts. None of the spots react with Neu's reagent nor ferric chloride. Their reaction with Pauly reagent (no color with solution I, brown with I + II) suggests flavonoids (IX) p-hydroxy benzoic acid (XV) or p-hydroxy phenyl alkyl derivatives (Hergert, 1960). They do absorb 360 nm UV light, suggesting flavonol glycosides (not very probable in this solvent), anthocyanins or flavones (Geissman, 1955).

No quantitative data were obtained on these fractions because all spots were rather small. Qualitative data, showing among-tree variation in all sampling groups, are presented in Tables 9 through 13. Compounds 4, 6, 9 and 10 are present in only one of seven American chestnut individuals as determined by thin-layer chromatography. Compound 5 was present in only two individuals, 7 in three trees and 8 was present in six of the trees sampled. Only compounds 1, 2, and 3 were found in all individual trees sampled (Figure 2, p. 45).

A pattern similar to that of American sound bark is also observed in the infected bark (Figure 3, p. 46) and twig (Figure 5, p. 48) extracts, although the variation is not quite so great. Only in the exotic sound bark and American root bark samples were all compounds

Table 9. Mean hR_F-values and qualitative variation among components of individual American chestnut sound inner bark extracts soluble in chloroform and chromatogrammed with benzene-methanol (90:10) on silica gel thin-layers

Spot	Mean hR_F-value	Tree number						
		6	7	8	9	10	11	12
1	20	+	+	+	+	+	+	+
2	22	+	+	+	+	+	+	+
3	26	+	+	+	+	+	+	+
4	37	-	-	-	-	-	-	+
5	52	-	-	-	-	-	+	+
6	88	-	-	-	-	-	-	+
7	92	-	-	-	-	+	+	+
8	98	+	-	+	+	+	+	+
9	60	-	-	-	+	-	-	-
10	66	-	-	-	+	-	-	-

+ = present; - = absent.

Table 10. Mean hR_F-values and qualitative variation among components of individual American chestnut infected inner bark extracts soluble in chloroform and chromatogrammed with benezene-methanol (90:10) on soluble gel thin-layers

Spot	Mean hR_F-value	Tree number		
		6	7	8
1	24	+	+	+
2	26	+	+	+
3	28	+	+	+
4	30	+	+	+
5	32	+	+	+
6	34	+	+	-
7	36	+	+	+
8	55	+	-	-
9	93	+	-	-

+ = present; - = absent.

Table 11. Mean hR_F-values and qualitative variation among components of individual American chestnut root inner bark extracts soluble in chloroform and chromatogrammed with benzene-methanol (90:10) on silica gel thin-layers

Spot	Mean hR_F-value	Tree number		
		9	10	12
1	23	+	+	+
2	27	+	+	+
3	36	+	+	+
4	98	+	+	+

+ = present; - = absent.

Table 12. Mean hR_F-values and qualitative variation among components of individual American chestnut debudded twig extracts soluble in chloroform and chromatogrammed with benzene methanol (90:10) on silica-gel thin-layers

Spot	Mean hR_F-values	Tree number				
		7	9	10	11	12
1	5	-	+	-	+	-
2	22	+	+	+	+	+
3	25	+	+	+	+	+
4	27	+	+	+	+	+
5	31	+	+	+	+	+
6	33	+	+	+	+	+
7	35	+	+	+	+	+
8	42	-	+	-	+	+
9	68	+	+	-	+	+
10	93	-	+	-	-	-
11	98	-	+	-	-	+

+ = present; - = absent.

Table 13. Mean hR_F-values and qualitative variation among components of individual exotic chestnut sound inner bark extracts soluble in chloroform and chromatogrammed with benezene-methanol (90:10) on silica gel thin-layers

Spot	Mean hR_F-value	Tree number		
		20	21	22
1	22	+	+	+
2	32	+	+	+
3	40	+	+	+

+ = present; - = absent.

present in every tree. These two sampling solubility groups are singularly notable, however, for the simplicity of their chromatographic patterns (Figure 6, p. 49, and Figure 4, p. 47, respectively).

Ether-Soluble Fractions

Only extracts from American chestnut trees 6, 7 and 8, which were the first extracted, show signs of containing phenolic compounds, judged from results of treating thin-layer chromatograms with ferric chloride-potassium ferricyanide reagent. This observation was made in spite of the very large (up to 25 µl) samples of some extracts applied and chromatogrammed as compared with the standard 2 µl samples. As a result, only chromatograms of ether-soluble extracts from American chestnut sound and infected stem inner bark and twigs were available for comparison. No American chestnut root or exotic chestnut inner bark extract yielded a discernible chromatographic pattern, nor did sound bark or twig samples from American chestnut trees 9 through 12. A possible explanation for this anomaly is the use of diethyl ether from

a container which had been opened for some time in extracting samples from American chestnut trees 6 through 8. A new can of ether was used to extract the later American chestnut samples (9 through 12) and the exotic material. It is possible that earlier extracts were made with ether containing water adsorbed from the atmosphere while later extracts were made with anhydrous ether. Since all available bark material from trees 6, 7 and 8 was used in earlier extractions, it was not possible to extract a portion of this material with anhydrous ether. A second extract of some material collected later (American chestnut 9 through 12; exotics), refluxed in the Soxhlet for two days with anhydrous ether failed to yield phenolic compounds on thin-layer chromatograms.

Of course, it is possible that none of the material collected and extracted later contained phenolic compounds soluble in ether. Trees 6, 7 and 8 all came from the same general location (Table 2, p. 27) and could have some components in other solubility groups not present in trees from other locations. Since this possibility seems highly unlikely, it would appear reasonable to expect the acetone- and methanol-soluble extractives of samples collected and extracted later to consistently have more component spots than those of the earlier extractives; at least some of the compounds not extracted in ether should be removed by one of these solvents. This would be especially true if the first hypothesis, concerning water vapor in the ether, were true. Careful screening of chromatograms of these two fractions did not, however, disclose consistent differences, in the form of extra component spots, from the earlier samples (6, 7 and 8) from which ether-soluble phenolics were obtained.

Egger (1969) suggests that diethyl ether, in the sequential extraction procedure used, removes phenolic aglycones and a few glycosides (rhamnosides). As in the case of the chloroform-soluble extracts, it was necessary to turn to a solvent recommended for anthraquinone glycosides (Stahl and Schorn, 1969), benzene-acetic acid (66:33), and alter it to the proportions 85:15 in order to obtain satisfactory chromatographic results.

Composite thin-layer chromatograms of American chestnut sound and infected inner bark and twigs are presented in Figures 8, 9, and 10, respectively. Included in the figures are mean hR_F-values and color reactions of component spots when treated with chromophoric reagents.

Spots which could represent the same compound, as determined by comparing mean hR_F-values with $\pm 2S\bar{x}$ and their color reactions, are presented in Table 14. Spots 2 and 3 of the sound and infected bark extracts, respectively, could be the same, as could 3 and 5 of the same material. Spot 1 of the infected bark extracts corresponds to spot 1 of the twig extract.

None of the spots in any of the three extracts produced a reaction with Neu's reagent. While this probably does not rule out flavonoid compounds completely, it suggests that the likelihood of any compounds of this class being present is small.

Spots 1 of the sound bark extract, 1 and 3 of the infected bark extracts and 2 of the twig extracts are a reddish brown with diazotized-4-nitroanaline, suggesting phenolic acids (Hathway, 1969). Reactions with other reagents are non-conclusive, some reinforcing and some

Spot No.	Mean hR_F	Color with: 1	2	3	4	5	6	7
3	93	-	-	f-Y	vf-Y	vf-Y	f-Y	-
2	71	-	-	-	-	-	f-Y	-
1	68	vf-Y	-	f-Y	-	-	f-Pk	-

Figure 8. Composite thin-layer chromatogram of ether-soluble fractions of American chestnut sound inner bark. Chromatogrammed on Eastman Chromagram sheet, type 6061 silica gel with benzene-acetic acid (85:15) solvent; 12 cm migration. All spots positive with $FeCl_3$-$K_3Fe(CN)_6$ reagent. Other reagents: (1) visible light, (2) 2% $FeCl_3$ in 0.5N HCl, (3) tetrazotized benezidine, (4) Pauly reagent I, (5) Pauly reagent I + II, (6) diazotized 4-nitroanaline, (7) Neu's reagent + 360 nm UV light.

Y = Yellow; Pk = Pink.
v = very; f = faint.

Spot No.	Mean hR_F	Color with: 1	2	3	4	5	6	7
5	94	-	-	1Bn	vf-Y	-	1Bn	-
4	73	-	-	-	-	vf-Bn	1-Y	-
3	68	-	-	Y	-	vf-Bn	R-Bn	-
2	21	-	-	1-R	Y	f-Y	Y	-
1	04	f-Bn	Gy-B	f-Or	Y	-	1R-Bn	-

Figure 9. Composite thin-layer chromatogram of ether-soluble fractions of infected American chestnut inner bark. Chromatogrammed on Eastman Chromagram sheet, type 6061 silica gel with benzene-acetic acid (85:15) solvent; 12 cm migration. All spots positive with $FeCl_3$-$K_3Fe(CN)_6$ reagent. Other reagents: (1) visible light, (2) 2% $FeCl_3$ in 0.5N HCl, (3) tetrazotized benzidine, (4) Pauly reagent I, (5) Pauly reagent I + II, (6) diazotized 4-nitroanaline, (7) Neu's reagent + 360 nm UV light.

R = Red; Bn = Brown; Y = Yellow; Gy = Gray.
f = faint; v = very; l = light.

Spot No.	Mean hR_F	Color with: 1	2	3	4	5	6	7
4	97	-	-	1-Bn	vf-Y	-	1-Bn	-
3	79	Y-Gn	-	-	-	-	f-Bn	-
2	73	Y-Gn	f-Bn	Y	vf-Y	vf-Bn	1-BBn	-
1	05	f-Bn	Gy-B	-	f-Bn	vf-Bn	1-Bn	-

Figure 10. Composite thin-layer chromatogram of ether-soluble fractions of American chestnut twigs. Chromatogrammed on Eastman Chromagram sheet, type 6061 silica gel with benzene-acetic acid (85:15) solvent; 12 cm migration. All spots positive with $FeCl_3$-$K_3Fe(CN)_6$ reagent. Other reagents: (1) visible light, (2) 2% $FeCl_3$ in 0.5N HCl, (3) tetrazotized benzidine, (4) Pauly reagent I, (5) Pauly reagent I + II, (6) diazotized 4-nitroanaline, (7) Neu's reagent + 360 nm UV light.

Y = Yellow; Gn = Green; Gy = Gray; B = Black; Bu = Brown.
v = very; f = faint; l = light.

Table 14. Spots which could represent the same compounds, found in ether-soluble extracts from American chestnut sound and infected inner bark and debudded twigs. Similarity established by comparing mean hR_F values with ±2 standard errors and color reactions.

Sampling Group		
American Sound	American Infected	American Twigs
2	2	-
3	5	-
-	1	1

conflicting with this result. All four of these spots do fluoresce yellow in 360 nm UV light, suggesting anthraquinone derivatives (Stahl and Schorn, 1969).

Qualitative data for the ether-soluble fractions is presented in Tables 15 through 17. Spot 1 of the American sound bark extract was not observed in tree 7. Infected bark extract displayed a similar pattern of variation, but this could be a reflection of sample size, as the infected bark sample from tree 6 was 13 grams while for 7 and 8 it was only 10 and 5.7 grams, respectively (Table 2, p. 27).

Acetone-Soluble Fractions

The stepwise extraction procedure used removes most of the phenolic glycosides in the acetone and methanol fractions (Egger, 1969). These fractions were found to contain by far the greatest number of components, and both required two-dimensional chromatography to achieve satisfactory separation of components. First-direction solvent used for

Table 15. Mean hR_F-values and qualitative variation among components of individual American chestnut sound stem inner bark extracts soluble in diethyl ether and chromatogrammed with benzene-acetic acid (85:15) on silica gel thin-layers

Spot	Mean hR_F-value	Tree number		
		6	7	8
1	68	+	-	+
2	71	+	+	+
3	93	+	+	+

Table 16. Mean hR_F-values and qualitative variation among components of individual American chestnut infected stem inner bark extracts soluble in diethyl ether and chromatogrammed with benezene-acetic acid (85:15) on silica gel thin-layers

Spot	Mean hR_F-value	Tree number		
		6	7	8
1	4	+	+	-
2	21	+	+	+
3	68	+	-	-
4	73	+	+	+
5	94	+	+	+

Table 17. Mean hR_F-values and qualitative variation among components of individual American chestnut debudded twig extracts soluble in diethyl ether and chromatogrammed with benezene-acetic acid (85:15) on silica gel thin-layers

Spot	Mean hR_F	Tree number
		7
1	5	+
2	73	+
3	79	+
4	97	+

the acetone-soluble fractions was one recommended for anthraquinone glycosides; ethyl acetate-methanol-water (100:16.5:13.5) (Stahl and Schorn, 1969). After drying 30 minutes in a hood, n-butanol-acetic acid-water (BAW) (63:10:27) (Hathway, 1969), was applied in the second direction.

Acetone-soluble fractions of the bark extracts were most rewarding in terms of the variety of colors obtained upon treatment with the various chromophoric reagents. Both acetone and methanol solubility groups provide an excellent opportunity to study qualitative and quantitative variation among individual trees within sampling groups and for comparisons among sampling groups in an attempt to discover differences and similarities.

Composite two-dimensional thin-layer chromatograms of acetone-soluble fractions of American chestnut sound, infected and root inner bark and debudded twigs and exotic chestnut inner bark are presented in Figures 11 through 15, respectively. Colors observed with Pauly reagent (diazotized sulfanilic acid) are also noted.

Several spots of the acetone-soluble extracts yielded reddish-pink colors with Pauly reagent (Table 18). Some of these components also produced a yellow color with solution I alone.

Hergert (1960) found that derivatives of guaiacol without an alpha hydroxyl or carbonyl broup (XVI) gave a pink color with this reagent, and that only catechins (III, p. 20), leucocyanidins (IV, p. 20) and phloroglucinol (VIII, p. 43) gave a yellow color with solution I alone. The chemical structures possible for these component spots are, therefore, limited. Because catechins and leucocyanidins should produce

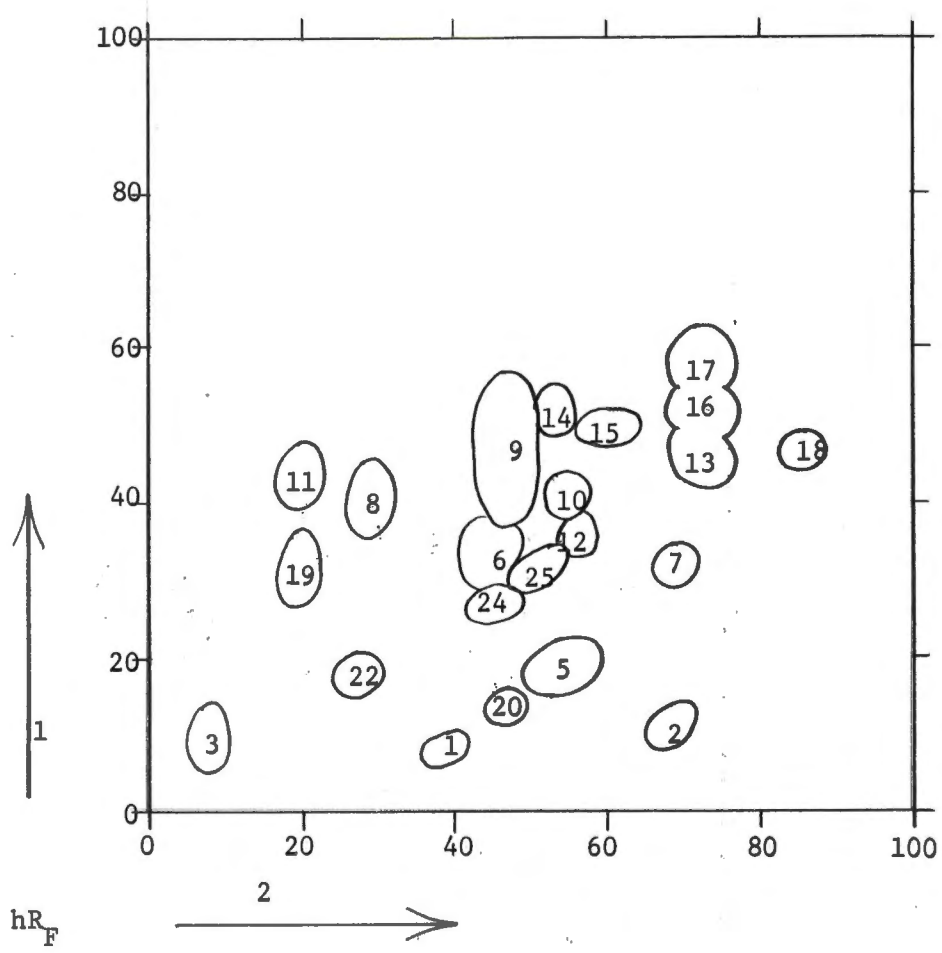


Figure 11. Composite two-dimensional silica gel thin-layer chromatogram of acetone soluble extracts from American chestnut sound inner bark. All spots positive with $FeCl_3$-$K_3Fe(CN)_6$ reagent. Solvents: 1-Ethyl acetate-methanol-water (100:16.5:13.5), 2-n-Butanol-acetic acid-water (63:10:27). Colors with diazotized sulfanilic acid: Pink to Red--2, 5, 7, 12, 18, 20, 23; Orange--8, 10, 13, 14, 16, 17, 19; Yellow-Green to Brown--1, 3, 6, 9, 11, 12, 15, 19, 22.

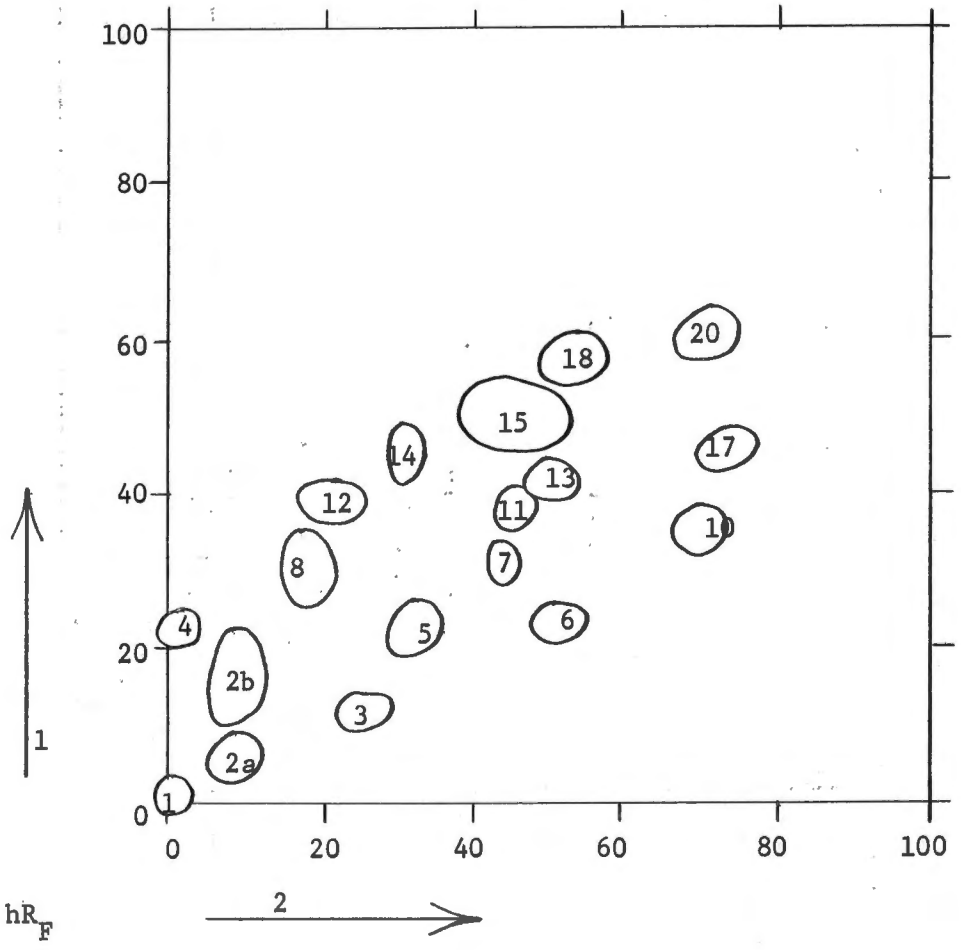


Figure 12. Composite two-dimensional silica gel thin-layer chromatogram of acetone soluble extracts from American chestnut infected inner bark. All spots positive with $FeCl_3$-$K_3Fe(CN)_6$ reagent. Solvents: 1-Ethyl acetate-methanol-water (100:16.5:13.5), 2-n-Butanol-acetic acid-water (63:10:27). Colors with diazotized sulfanilic acid: Pink to Red--6, 13, 19; Yellow-Green to Brown--all others.

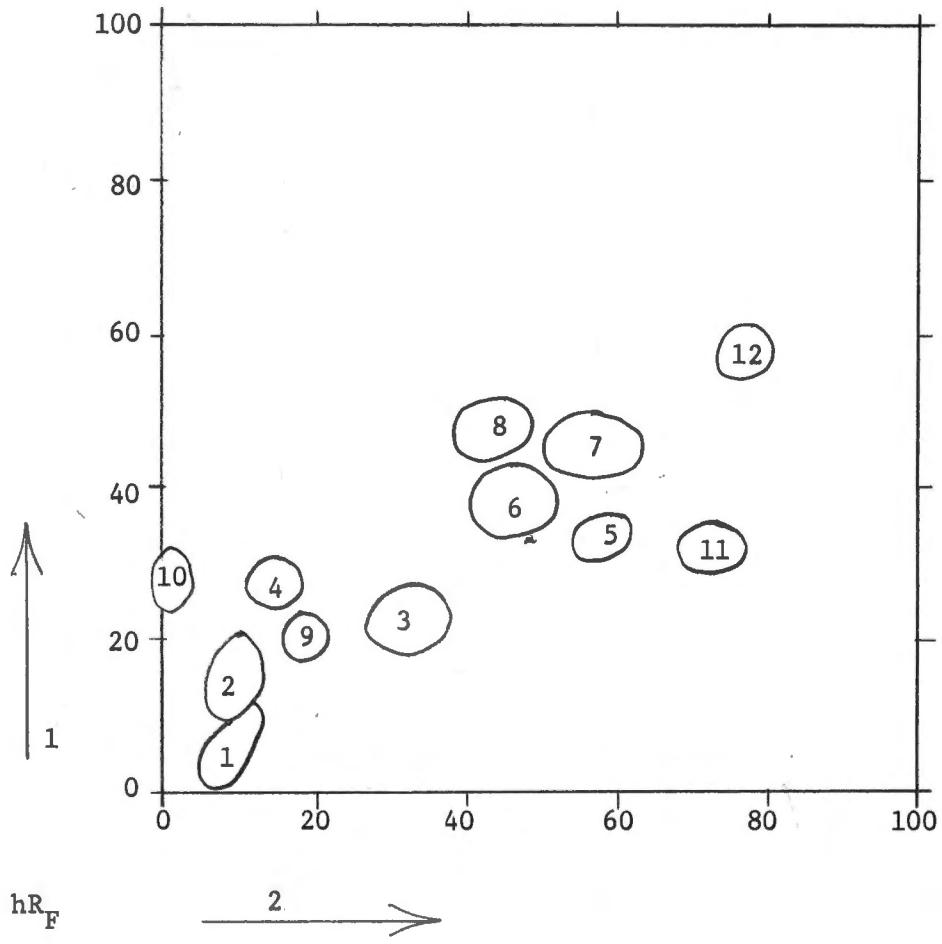


Figure 13. Composite two-dimensional silica gel thin-layer chromatogram of acetone-soluble extract of American chestnut sound root inner bark. All spots positive with $FeCl_3$-$K_3Fe(CN)_6$ reagent. Solvents: 1-Ethyl acetate-methanol-water (100:16.5:13.5), 2-n-Butanol-acetic acid-water (63:10:27). Colors with diazotized sulfanilic acid: Orange--3, 7; Yellow-Green to Brown--1, 2, 4, 5, 6, 8, 9, 10, 11.

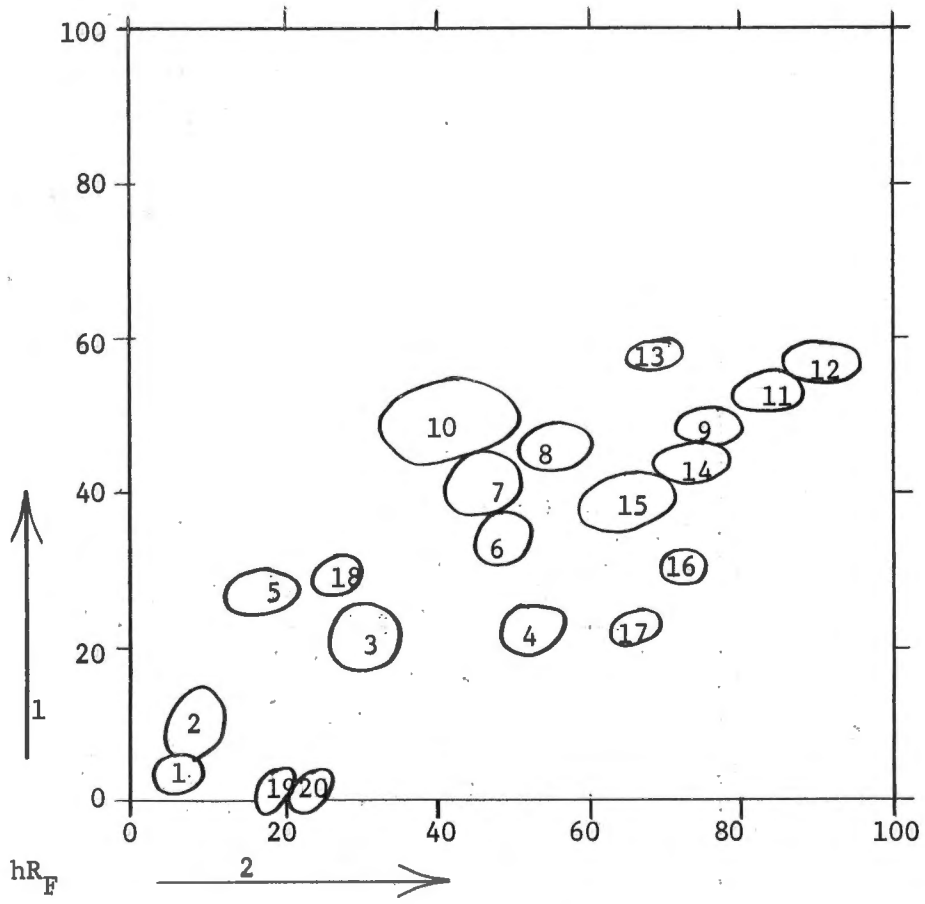


Figure 14. Composite two-dimensional silica gel thin-layer chromatogram of acetone-soluble extracts from American chestnut debudded twigs. All spots positive with $FeCl_3$-$K_3Fe(CN)_6$ reagent. Solvents: 1-Ethyl acetate-methanol-water (100:16.5:13.5), 2-n-Butanol-acetic acid-water (63:10:27). Colors with diazotized sulfanilic acid: Pink to Red--4, 9, 11, 12; Orange--3; Brown--all others.

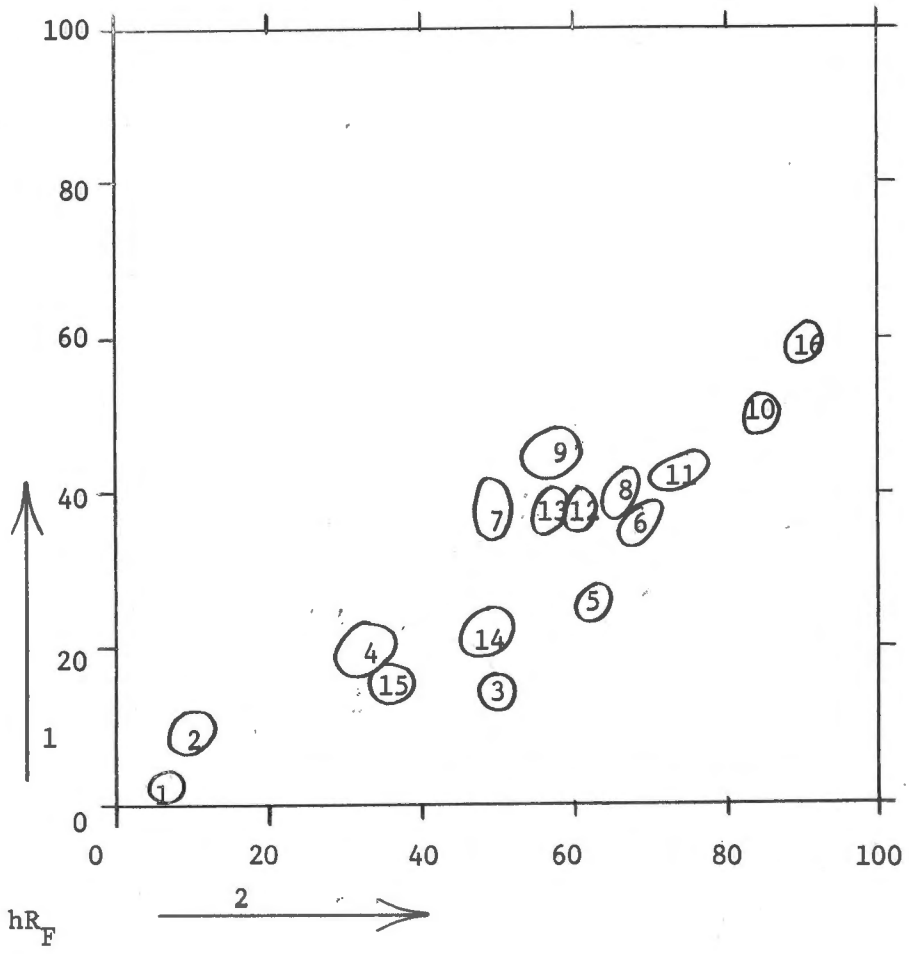


Figure 15. Composite two-dimensional silica gel thin-layer chromatogram of acetone soluble extracts from exotic chestnut inner bark. All spots positive with $FeCl_3$-$K_3Fe(CN)_6$ reagent. Solvents: 1-Ethyl acetate-methanol-water (100:16.5:13.5), 2-n-Butanol-acetic acid-water (63:10:27). Colors with diazotized sulfanilic acid: Pink to Red--→3, 5, 6, 8, 10 through 16; Brown--1, 2; Yellow--4, 7, 9.

Table 18. Acetone-soluble inner bark components from Castanea which yield pink to red colors with Pauly reagent on silica gel thin-layer chromatograms

American Sound	American Infected	American Twig	Exotic	
2[a]	6	4[a]	3	12
5[a]	13[a]	9[a]	5	13
7[a]	19	11	6[a]	14
12[a]		12[a]	8[a]	15
18			10[a]	16
20[a]			11	
23[a]				
Fig. 11	Fig. 12	Fig. 14	Fig. 15	

[a]Yellow with solution I only.

a yellow color with solutions I + II, these possibilities are also eliminated, leaving only the possibility of derivatives of guaiacol without an alpha hydroxyl or carboxyl group or phloroglucinol.

Orange colors were produced by some spots when treated with Pauly reagent (Table 19). Some of these turned brown-yellow to orange with solution I alone. This combination of reactions suggests guaiacol (XVI, p. 52), alpha-hydroxy guaiacol or vanillic acid (XV, p. 52) derivatives (Hergert, 1960). The same spots turned a deep orange-red with tetrazotized benzidine. Hathway (1969) found that red colors with this reagent are indicative of phenols of the phloroglucinol- (VIII, p. 43) resorcinol-type. In order to fill both these criteria there must be meta hydroxyl substitution in the ring system.

The majority of spots from all sampling groups exhibited various shades of yellow-green to brown following treatment with Pauly reagent (Table 20); some exhibited color with solution I alone.

Table 19. Acetone-soluble inner bark components from Castanea which yield an orange color with Pauly reagent on silica gel thin-layer chromatograms

American Sound	American Root	American Twig
8[a] 16[a]	3	3
10 17[a]	7	
13[a] 19		
14		
Fig. 11, p. 66	Fig. 13, p. 68	Fig. 14, p. 69

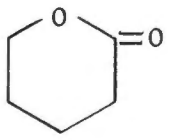
[a]Any color with solution I alone.

Table 20. Acetone-soluble inner bark components from Castanea which yield yellow-green to brown colors with Pauly reagent on silica gel thin-layer chromatograms

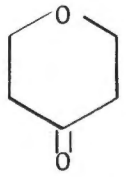
American Sound	American Infected	American Root	American Twig	Exotic
1[a]	1[a] 10[a]	1[a]	1[a] 15[a]	1[a]
3[a]	2a[a] 11[a]	2[a]	2[a] 16[a]	2[a]
6[a]	2b[a] 12[a]	4[a]	5 17[a]	4[a]
9[a]	3[a] 14[a]	5[a]	6[a] 18[a]	7[a]
11	4[a] 15[a]	6[a]	7[a] 19[a]	9[a]
12	5[a] 16[a]	8[a]	8[a] 20[a]	
15	7[a] 18[a]	9[a]	10[a]	
19	8[a] 19[a]	10[a]	13[a]	
22	9[a] 20	11	14[a]	
Fig. 11, p. 66	Fig. 12, p. 67	Fig. 13, p. 68	Fig. 14, p. 69	Fig. 15, p. 70

[a]Any color with solution I alone.

Solution I of Pauly reagent was found to form colors with catechins (III, p. 20), leucocyanidins (IV, p. 20) and phloroglucinol (VIII, p. 43) (Hergert, 1960). Among the compounds just listed, spots 6 and 9 of the American sound inner bark and 15 of the American infected inner bark extracts also fluoresced in 360 nm UV light following treatment with Neu's natural product reagent. This reaction is characteristic of compounds having the alpha (XX) or gamma (XXI) pyrone group



XX



XXI

of the coumarin or flavonoid nucleus (Randerath, 1966). Catechins and leucocyanidins belong to the flavonoid class of compounds, having the basic C_6-C_3-C_6 structure, but being the most reduced of the class they lack the gamma C=0 group in the pyrone ring (Robinson, 1967).

Since Hergert (1960) did not discuss a group yielding brown colors with Pauly reagent, it was necessary to apply his criteria for yellow colors to these components which were varying shades of yellow-green to brown. This would place these components in the category of flavonoid (IX, p. 43) or derivatives of p-hydroxy benzoic acid (XV, p. 52) or p-hydroxy phenyl alkyls. The probability of benzoic acid derivatives is diminished, however, because tetrazotized benzidine is said to yield rose-pink spots with phenolic acids (Hathway, 1969). Tetrazotized benezidine did not yield pink colors with any component separated in any solubility group nor with any standards, although standards

chromatogrammed included gallic, salicylic, chlorogenic and caffeic acids. This observation would cause one to question this reagent as being specific for phenolic acids.

Spots not covered by the discussion to this point were present in such low quantity that they did not react with the chromophoric reagent and could only be identified as phenolic by their reaction with ferric chloride-potassium ferricyanide.

Components of acetone-soluble extracts from American chestnut sound, infected and root inner bark and debudded twigs and exotic chestnut inner bark which could represent the same compounds are presented in Table 21. Only three spots located on chromatograms of American chestnut infected bark extracts corresponded with component spots of extracts from sound bark of the same tree. Spots 2a and 2b of the infected bark extracts exhibit no corresponding components in sound bark extracts. These spots were characteristically dark brown to almost black when first treated with Pauly reagent, later fading to dark brown. Other differences immediately apparent are the dramatic decrease in the size of component spot 9 of sound bark extracts, shown to correspond with spot 15 of the infected bark extracts (Figures 11 and 12, pp. 66 and 67, respectively), and the complete disappearance of the prominent orange-staining spots (13, 16 and 17) of the sound bark extracts from the infected bark extracts. The question is whether the Endothia enzyme system metabolizes these spots, the by-products being spots 2a and 2b of the infected bark extracts, or the change takes place simply as a reaction to the wounding caused by infection. Other differences were noted in chromatographic patterns of American chestnut

Table 21. Components of acetone-soluble extracts from American chestnut sound, infected and root inner bark and debudded twigs and exotic chestnut inner bark which could be the same compounds as determined by comparing mean hR_F ranges and color reactions

Sampling Group				
American Sound	American Infected	American Root	American Twig	Exotic Sound
3	–	–	–	2
–	2a	1	–	–
6	–	–	–	–
9	15	8	10	–
11	12	–	–	–
12	–	7	8	9
17	–	–	13	–
18	–	–	11	10
25	13	–	–	7
–	6	–	–	14
–	11	6	7	7
–	–	6	6	–
–	–	–	2	2
–	17	–	–	11
–	2b	2	–	–
–	8	4	5	–
–	–	3	3	–
–	–	–	1	1

sound and infected inner stem bark; those discussed are only the most prominent changes.

In this connection, two spots of the acetone-soluble root extracts from American chestnut correspond with spots of infected bark extracts (Table 21). These components could be of significance in resistance to Endothia in American chestnut, since the root system is apparently not attacked by the fungus.

Only two spots of the American chestnut root inner bark extracts and five spots of the American chestnut twig extracts correspond with sound stem bark extracts (Table 21). It will be recalled that spot 9 of the American chestnut sound bark extracts is the most prominent found in this group. This spot corresponds with spot 15 in infected bark extracts and also with spot 10 of twig extracts. If this component is metabolized by the fungus and it were found to be possible to increase resistance to the blight fungus in American chestnut trees by selecting for less of this component, then twig extracts could be used effectively as an indicator in the selection of resistant trees. The same reasoning would hold for spots 11-twig and 18-sound.

Only spots 7, 9 and 10 of exotic chestnut inner bark extracts correspond with spots 25, 12 and 18, respectively, of American chestnut sound inner bark extracts (Table 21). It appears, therefore, that the use of acetone-soluble compounds found in exotic chestnut extracts as a guide to selection of American chestnut individuals resistant to Endothia parasitica holds little promise unless it can be shown that component 7, 9, or 10 (or a combination of them) is contributing to the resistance observed in exotic chestnut. Component spots 7, 9,

and 10 of exotic chestnut extracts were present in all trees sampled; no quantitative variation was noted. Conversely, in the case of American chestnut sound stem inner bark extracts, spot 25 was present in only three of the seven trees sampled, while spots 12 and 18 were noted in six and four trees, respectively. Quantitative variation was also noted among samples exhibiting these three spots.

A total of seven individual American chestnut trees were sampled for sound inner bark. Quantitative and qualitative variation among the acetone-soluble components of these bark samples is presented in Table 22, with mean hR_F-values for each component spot. The total number of components found in an individual tree varied from 11 (tree 6) to 18 (trees 9 and 10). Since a total of 25 component spots were discovered, no single tree had all components. Spots 3, 5, 6, 7, 8, 9, 14 and 17 were present in all trees sampled, while spots 2 and 19 were present in six of the seven trees sampled. Quantitative as well as qualitative variation was noted among trees in components 2, 11, 15, 18, 19 and 25, while only quantitative variation was noted in component 14.

Three American chestnut trees (6, 7 and 8) were sampled for infected inner bark. A total of 18 component spots were located, but no single tree contained all of them. Tree number 6 exhibited a total of 14 phenolic components while trees 7 and 8 had 13 and 11 such components, respectively (Table 23). Components 1, 2a, 2b, 4, 5, 10 and 15 were present in every tree sampled, but the amount of components 1 and 5 present varied among the trees sampled. Components 3, 9, 14 and 18 were present in only one of the trees sampled, while spots 6, 8, 12, 13

Table 22. Mean hR_F-values with qualitative and semi-quantitative variation in components of American chestnut sound inner bark soluble in acetone as determine by spot size on two-dimensional silica gel thin-layer chromatograms

Spot No.	Mean hR_F-value in:[a] 1	2	Tree number 6	7	8	9	10	11	12
1	7	39	-	+	+	+	-	-	-
2	10	68	-	++	+	+	+	+	+
3	9	8	++	++	++	++	++	++	++
5	19	54	++	++	++	++	++	++	++
6	31	45	++	++	++	++	++	++	++
7	32	69	++	++	++	++	++	++	++
8	39	29	++	++	++	++	++	++	++
9	46	46	+++	+++	+++	+++	+++	+++	+++
11	44	20	-	++	+	-	-	-	++
12	42	55	-	+	++	++	+	++	+
13	46	73	-	++	++	-	++	-	++
14	51	54	++	++	+	+	++	+	+
15	48	60	-	++	+	+	+	-	-
16	52	74	-	++	++	-	++	-	++
17	57	72	++	++	++	++	++	++	++
18	47	85	+	-	-	+	++	++	-
19	30	19	++	++	++	++	+	-	++
20	14	46	+	-	+	-	-	+	-
22	18	28	-	-	-	+	+	-	-
24	25	46	-	-	-	+	+	+	-
25	32	51	-	-	-	+	++	+	-
Bark sample (gm)			10	10	10.6	6.7	10	10	10
Chromatogrammed sample (µl)			6	6	5	4.2	6	6	6

[a] 1. Ethyl acetate-methanol-water (100:16.5:13.5)
2. n-Butanol-acetic acid-water (63:10:27)

- = Absent; + = <30 mm^2; ++ = 30 mm^2 to 200 mm^2; +++ = >200 mm^2.

Table 23. Mean hR_F-values with qualitative and semi-quantitative variation in components of American chestnut infected inner bark soluble in acetone as determined by spot size on two-dimensional silica gel thin-layer chromatograms

Spot No.	Mean hR_F value in:[a] 1	2	Tree number 6	7	8
1	0	0	+	+	++
2a	6	8	++	++	++
2b	16	8	++	++	++
3	12	24	-	-	++
4	22	1	+	+	+
5	23	33	++	+	+
6	22	51	+	+	-
8	30	18	-	++	++
9	32	44	+	-	-
10	34	69	++	++	++
11	38	46	++	-	-
12	40	22	-	++	+
13	41	49	-	+	++
14	45	32	++	-	-
15	50	47	++	++	++
17	45	73	++	++	-
18	57	53	+	-	-
20	60	72	++	++	-
Bark sample (gm)			13	10	5.7
Chromatogrammed sample (µl)			4	12	10

[a] 1. Ethyl acetate-methanol-water (100:16.5:13.5)
2. n-Butanol-acetic acid-water (63:10:27)

- = Absent; + = <30 mm^2; ++ = 30 mm^2 to 200 mm^2; +++ = >200 mm^2.

17 and 20 were present in two. Spots 12 and 13 varied quantitatively in two trees in which they appeared.

Three American chestnut trees (9, 10 and 12) were sampled for root bark (Table 24). Only tree number 9 had all twelve components found in this sampling/solubility group; tree 10 did not contain spots 5, 11 or 12, while tree 12 lacked spots 9 through 12. Quantitatively there was little variation noted; spot areas in all trees were generally 30 to 200 mm^2, the only exception being spots 9, 11 and 12 in tree 9 and spot 4 of tree 10, which showed areas of <30 mm^2.

Twig samples were obtained from three American chestnut trees (7, 9 and 11). One tree (11) had a total of 13 components and number 7 had 10 component spots (Table 25). Spots 5, 6 and 12 were absent from tree 9, while spot 15 was present in this individual and absent from the other two. Tree number 7 lacked spots 5, 6 and 7. There did not seem to be much quantitative variation among individual trees; only in the case of spot 11, which tree number 11 exhibited in greater quantity than the other two individuals, and spot 1, which was found in lesser quantity in tree 9 than in the other two trees, was there a discernible difference.

Less variation was noted among exotic chestnut bark sample chromatograms than among similar American chestnut samples (Table 26). This should be expected, since trees 20 and 22 are full sib progenies of a C. mollissima cross, and number 21 a C. mollissima hybrid. Tree number 22 did, however, exhibit one spot (number 13) not found in either of the two remaining trees. Components 3, 5, 8 and 13, found in trees 20 and/or 22 were not noted in tree 21 (the hybrid). Similarly,

Table 24. Mean hR_F-values with qualitative and semi-quantitative variation in components of American chestnut sound root inner bark soluble in acetone as determined by spot size on two-dimensional silica gel thin-layer chromatograms

Spot No.	Mean hR_F-value in:[a] 1	2	Tree number 9	10	12
1	4	7	++	++	++
2	14	9	++	++	++
3	22	32	++	++	++
4	27	15	++	+	++
5	33	58	++	-	++
6	37	47	++	++	++
7	44	56	++	++	++
8	46	44	++	++	++
9	20	18	+	++	-
10	27	1	++	++	-
11	32	72	+	-	-
12	57	76	+	-	-
Bark sample (gm)			10	10	10
Chromatogrammed sample (µl)			10	10	10

[a] 1. Ethyl acetate-methanol-water (100:16.5:13.5)
2. n-Butanol-acetic acid-water (63:10:27)

- = Absent; + = <30 mm^2; ++ = 20 mm^2 to 200 mm^2; +++ = >200 mm^2.

Table 25. Mean hR_F-values with qualitative and semi-quantitative variation in components of American chestnut debudded twigs soluble in acetone as determined by spot size on two-dimensional silica gel thin-layer chromatograms

Spot No.	Mean hR_F-value in:[a] 1	2	Tree number 7	9	11
1	3	6	++	+	++
2	9	8	++	++	++
3	21	30	++	++	++
4	23	53	++	++	++
5	27	16	-	-	++
6	34	50	-	-	+
7	41	47	-	++	++
8	46	55	++	++	++
9	48	76	++	++	++
10	49	42	++	+++	++
11	52	84	+	+	++
12	57	89	+	-	+
13	60	73	++	++	++
15	38	64	-	+	-
Bark sample (gm)			10	12.4	10
Chromatogrammed sample (µl)			15	12	15

[a] 1. Ethyl acetate-methanol-water (100:16.5:13.5)
2. n-Butanol-acetic acid-water (63:10:27)

- = Absent; + = <30 mm^2; ++ = 30 mm^2 to 200 mm^2; +++ = >200 mm^2.

Table 26. Mean hR_F-values with qualitative and semi-quantitative variation in components of exotic chestnut sound inner bark soluble in acetone as determined by spot size on two-dimensional silica gel thin-layer chromatograms

Spot No.	Mean hR_F-value in:[a] 1	2	Tree number 20	21	22
1	2	6	++	++	+
2	9	9	++	++	+
3	14	49	++	-	+
4	19	32	++	++	++
5	26	62	+	-	+
6	37	68	++	++	++
7	38	48	++	++	++
8	40	66	++	-	++
9	45	58	++	++	++
10	50	84	+	+	+
11	42	73	++	++	++
12	37	60	+	+	+
13	37	55	-	-	+
14	22	48	++	++	++
15	14	36	+	++	+
16	58	90	-	++	-
Bark sample (gm)			10	10	10
Chromatogrammed sample (µl)			15	15	15

[a] 1. Ethyl acetate-methanol-water (100:16.5:13.5)
2. n-Butanol-acetic acid-water (63:10:27)

- = Absent; + <30 mm^2; ++ = 30 mm^2 to 200 mm^2; +++ = >200 mm^2.

spot 16, found in tree number 21 was not found in the two C. mollissima individuals.

Little quantitative variation was noted; trees 20 and 21 exhibited slightly more of components 1 and 2 than did tree 22.

Methanol-Soluble Fractions

With the exception of American chestnut sound inner bark from roots, methanol-soluble fractions exhibited as great a diversity of component spots as was observed in the acetone-soluble fractions. This solubility group did not, however, exhibit the clarity and diversity of color reactions noted in acetone-soluble fractions. In some cases there was not the sharp separation of spots by comparison of mean hR_F-values with $\pm 2S_{\bar{x}}$ observed in the case of acetone-soluble fractions. As a result, several spots of a given sampling group could be possible matches for more than one spot in one or more other sampling groups. The primary cause is felt to be the use of 2% acetic acid as the first direction solvent. This was the only solvent of several tried which would provide separation of component spots in methanol-soluble fractions. The result was an irregular solvent front and hR_F-values which fluctuated somewhat. Separation with this solvent was not very efficient for some of the American root bark extracts; however, for comparison with other sampling groups it was used in spite of this limitation.

Composite two-dimensional thin-layer chromatograms of methanol-soluble fractions of American chestnut sound, infected and root inner bark and twigs and exotic chestnut inner bark are presented in Figures 16 through 20, respectively. Colors observed with Pauly reagent are also described in captions.

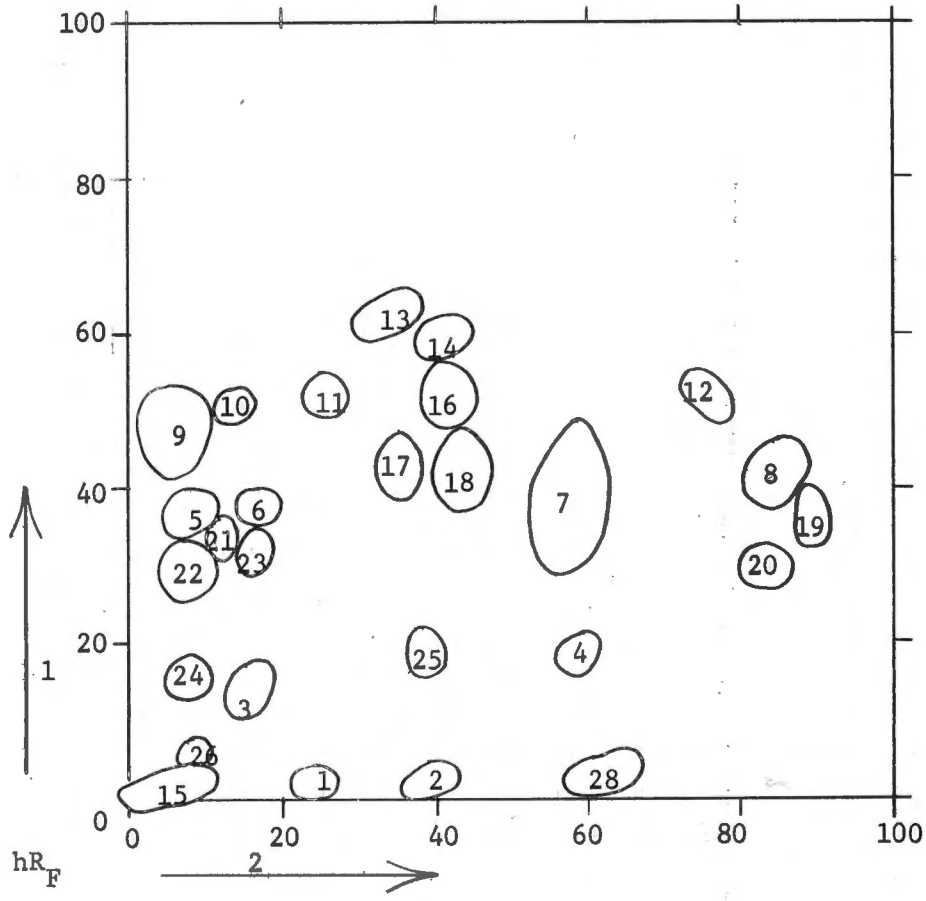


Figure 16. Composite two-dimensional silica gel thin-layer chromatogram of methanol-soluble extracts from American chestnut sound inner bark. All spots positive with $FeCl_3$-$K_3Fe(CN)_6$ reagent. Solvents: 1-2% acetic acid; 2-n-Butanol-acetic acid-water (63:10:27). Colors with Pauly reagent: Pink--13, 14; Brown--1, 2, 3, 5, 7, 9, 10; remainder not recorded.

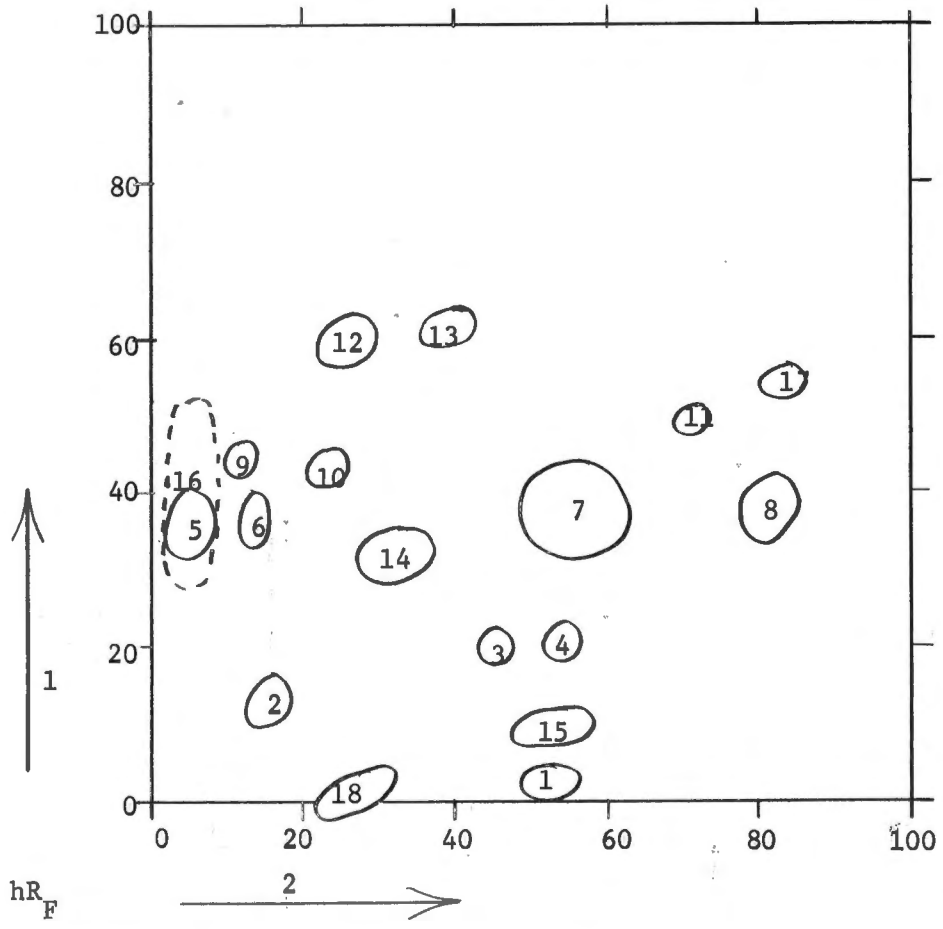


Figure 17. Composite two-dimensional silica gel thin-layer chromatogram of methanol-soluble extracts from American chestnut infected inner bark. All spots positive with $FeCl_3$-$K_3Fe(CN)_6$ reagent. Solvents: 1-2% acetic acid, 2-n-Butanol-acetic acid-water (63:10:27). Colors with Pauly reagent: faint Brown--7, 8; remainder not recorded.

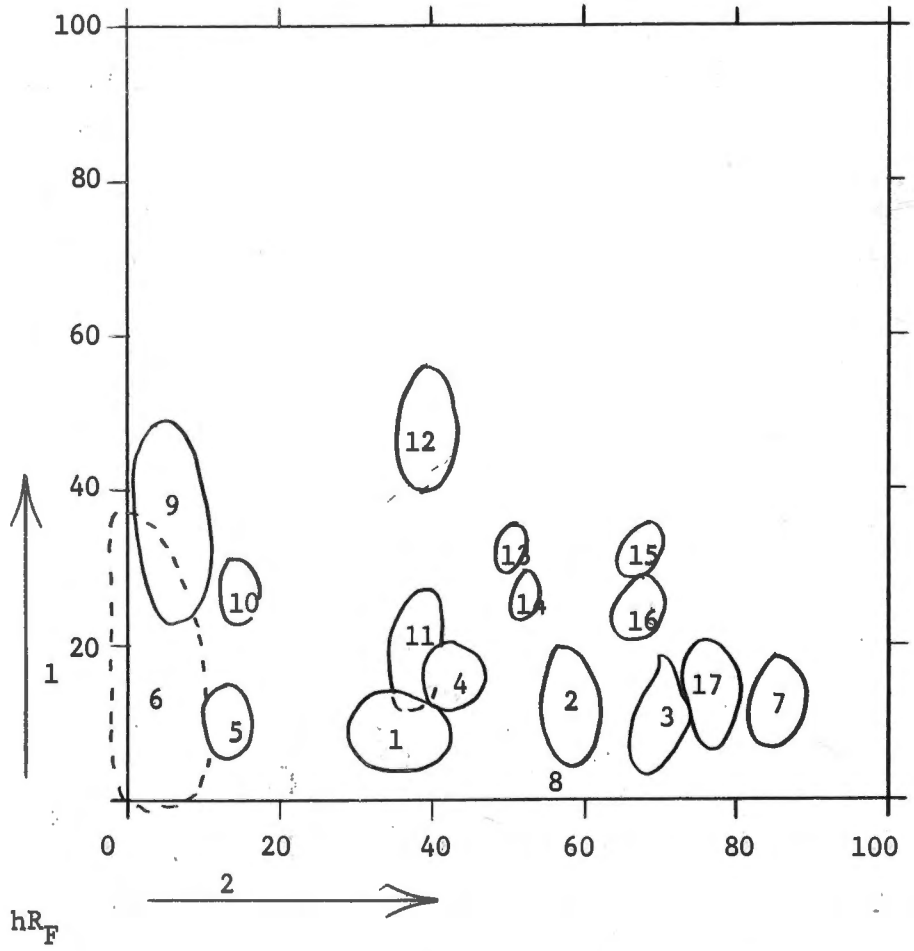


Figure 18. Composite two-dimensional silica gel thin-layer chromatogram of methanol-soluble extracts from American chestnut root inner bark, All spots positive with $FeCl_3$-$K_3Fe(CN)_6$ reagent. Solvents: 1-2% acetic acid; 2-n-Butanol-acetic acid-water (63:10:27). Colors with Pauly reagent: Orange-Brown--1, 4, 17; Dark Brown--2, 3, 5, 6; remainder not observed.

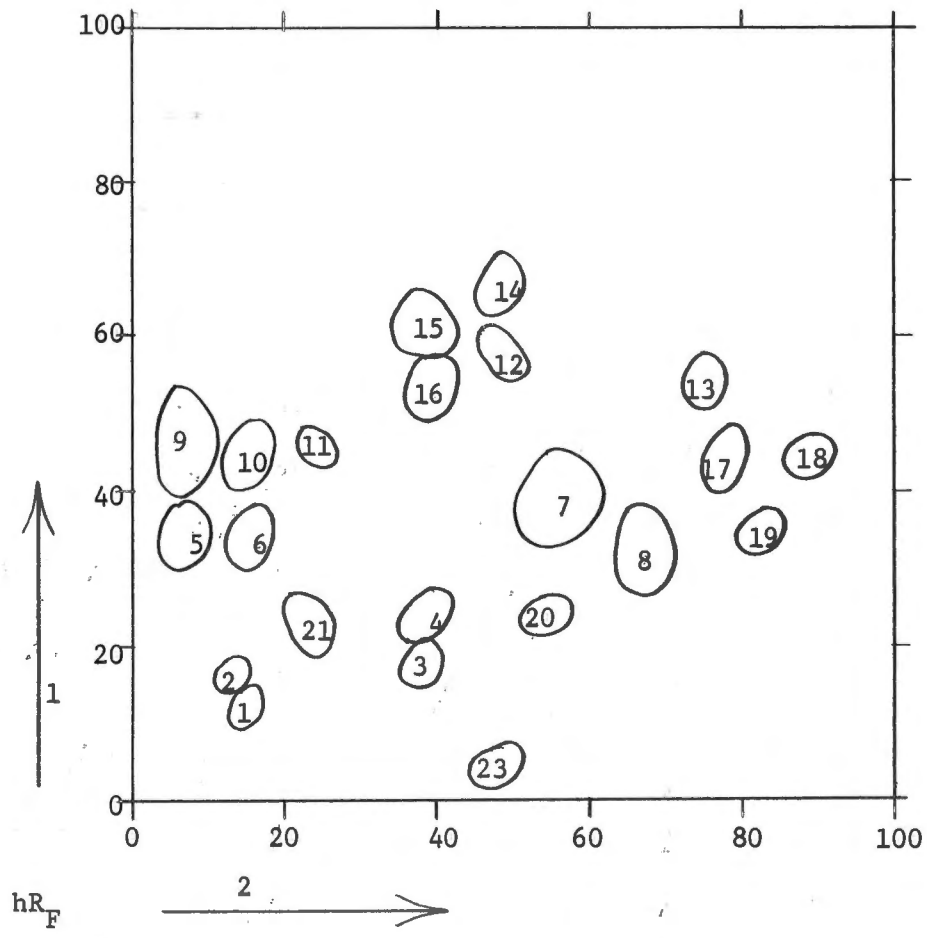


Figure 19. Composite two-dimensional silica gel thin-layer chromatogram of methanol-soluble extracts from debudded twigs of American chestnut. All spots positive with $FeCl_3$-$K_3Fe(CN)_6$ reagent. Solvents: 1-2% acetic acid, 2-n-Butanol-acetic acid-water (63:10:27). Colors with Pauly reagent: Orange-Brown--3, 4; faint Lavender--13; Pink--14; faint Brown--5 through 12.

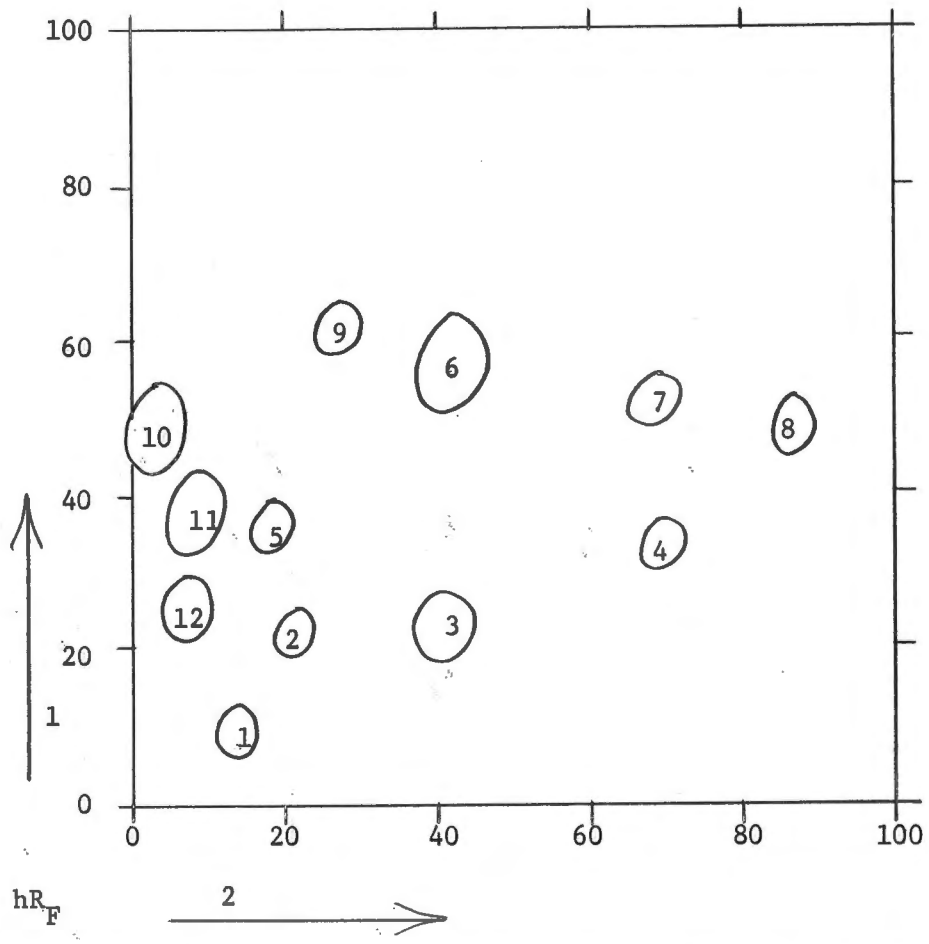


Figure 20. Composite two-dimensional silica gel thin-layer chromatogram of methanol-soluble extracts from exotic chestnut inner bark. All spots positive with $FeCl_3$-$K_3Fe(CN)_6$ reagent. Solvents: 1-2% acetic acid, 2-n-Butanol-acetic acid-water (63:10:27). Colors with Pauly reagent: light Brown--3, 6, 10, 11, 12; very faint Pink--9; remainder not observed.

Color data will not be presented for every spot in every sampling group with every color reagent. Due to the qualitative variation noted in the group this would have entailed the production of a chromatogram of the extract from almost every individual tree for treatment with every reagent. Colors were not very distinct with most reagents, so ferric chloride-potassium ferricyanide was used to the greatest extent.

Only a few spots yielded red to pink colors with Pauly reagent:

American Sound	American Twig	Exotic
13	14	9
14		
Fig. 16	Fig. 19	Fig. 20

This reaction suggests derivatives of guaiacol without an alpha hydroxy or carbonyl group (XVI, p. 52) (Hergert, 1960).

Tetrazotized benezidine yields canary yellow to golden brown spots with flavonoid compounds (IX, p. 43) having a resorcinol A-ring and pyrogallol B-ring (Roux and Maihs, 1960). Colors with solution I of Pauly reagent suggests catechins, leucocyanidins or phloroglucinol, while a brown-yellow color with both solutions indicates the possible existence of flavonones, p-hydroxy benzoic acid (XIV, p. 52) or p-hydroxy phenyl alkyl derivatives. Therefore, the combination of these reactions indicates presence of the flavonoid nucleus, probably highly substituted with alkyl groups. Component spots following this pattern are found in Table 27. A few of these spots also fluoresce in 360 nm UV light following treatment with Neu's natural product reagent, suggesting the presence of the alpha (XX, p. 72) or gamma (XXI, p. 72) pyrone group, such as found in coumarin or flavonoid derivatives (Randerath, 1966).

Table 27. Methanol-soluble inner bark components from Castanea which yield characteristic colors with tetrazotized benezidine and also produce color reactions with Pauly reagent I on silica gel thin-layer chromatograms

American Sound	American Infected	American Root	American Twig	Exotic
2	7[a]	1[a]	3	3
5		2[a]	4	6
7[a]		3[a]	7[a]	10
9		5	8	11
				12
Fig. 16, p. 85	Fig. 17, p. 86	Fig. 18, p. 87	Fig. 19, p. 88	Fig. 20, p. 89

[a]Fluoresces in 360 nm UV light after treatment with Neu's reagent.

Spot 17 of the American infected sampling group also fluoresced faintly following treatment with Neu's reagent. This component probably was not present in sufficient quantity to yield a positive reaction with the other reagents used. Spot 7 of the same group also produced a dark blue color in the presence of ferric chloride, adding to the probability of the flavonoid structure with a pyrogallol B-ring (Hergert, 1960).

Spots 5, 6, 9 and 10 of the American twig extracts yielded orange-brown spots with diazotized 4-nitroanaline. They also produced yellow-brown colors with solution I of Pauly reagent and orange-brown colors with solutions I + II. This combination of reactions eliminates the possibility of catechin or leucocyanidin nuclei, favoring the phloroglucinol nucleus. The compounds could, therefore, be derivatives of vanillic acid (XV, p. 52), guaiacol (XVI, p. 52) or alpha hydroxy guaiacol (Hergert, 1960).

The remaining spots did not yield clearly distinctive colors with the chromophoric reagents. Their qualitative and quantitative variation and comparative makeup among sampling groups were described according to their reactions with ferric chloride-potassium ferricyanide reagent.

Component spots which could represent the same compounds, as noted in American sound and infected bark samples are presented in Table 28. The number of components which could be the same, as determined by matching mean hR_F-values and color reactions, is considerably higher in this sampling group than in the acetone-soluble fractions. One cause of this difference is the previously noted lack of distinct color reactions in methanol fractions so that this parameter was not available to discriminate among components.

Seven spots found in chromatograms of infected bark samples correspond with spots of the American sound bark components (Table 28). It should be noted that, as in the case of the corresponding acetone-soluble extracts, the chromatographic pattern of infected bark samples differs from the pattern observed for sound bark samples, although not nearly so much as in the case of acetone-soluble extracts. Sound bark samples of trees 6 and 7 (Table 29) have only four and three components, respectively, while tree number 8 has 15. In the corresponding infected bark samples there are 9, 6 and 17 component spots (Table 30). In order to discuss these extracts all component spots numbered 15 and above in the American sound bark extracts must be excluded because in the sound bark they are present only in trees 9 through 12. If the chromatograms are compared in this light the only great difference between sound and infected bark extracts is the absence of component

Table 28. Components of methanol-soluble extracts from American chestnut sound, infected and root inner bark and debudded twigs and exotic chestnut inner bark which could be the same compounds determined by comparing mean hR_F-ranges and color reactions

Sampling group				
American Sound	American Infected	American Root	American Twig	Exotic Sound
3	2	-	2	-
4	4	-	-	-
5	5	-	5	11
6	9	-	6	-
6	9	-	10	-
7	7	-	7	-
8	8	-	17	-
9	-	-	9	10
10	-	-	10	-
13	-	-	15	-
13	-	-	16	-
14	13	-	12	-
14	13	-	15	-
14	13	-	16	-
16	-	-	-	6
16	-	-	12	-
16	-	-	15	-
16	-	-	16	-
28	-	-	23	-
-	1	-	23	-
-	2	-	1	1
-	3	-	-	3
-	3	-	20	4
-	7	-	7	-
-	9	-	9	-
-	12	-	-	9
-	13	-	15	-
-	13	-	15	-
-	13	-	16	-
-	17	-	-	8
-	-	-	3	3
-	-	-	3	4
-	-	-	18	8
-	-	-	21	2

Table 29. Mean hR_F-values with qualitative and semi-quantitative variation in components of American chestnut sound inner bark soluble in methanol as determined by spot size on two-dimensional silica gel thin-layer chromatograms

Spot No.	Mean hR_F-value in:[a] 1	2	Tree number 6	7	8	9	10	11	12
1	2	23	–	–	++	–	–	+	–
2	2	38	–	–	++	–	–	–	–
3	14	15	++	++	+++	++	++	++	++
4	19	58	–	–	+	–	–	+	–
5	37	7	–	–	++	+	++	++	++
6	38	16	–	–	++	+	–	++	++
7	37	57	++	++	++	+++	+++	+++	++
8	42	84	++	++	++	–	++	++	++
9	49	5	–	–	++	–	–	++	–
10	51	13	–	–	++	–	–	–	–
11	52	25	–	–	+	–	–	–	–
12	52	75	–	–	++	–	–	–	–
13	63	34	++	–	+	–	++	++	++
14	60	40	–	–	++	–	–	++	–
15	1	5	–	–	++	–	–	++	++
16	51	40	–	–	–	++	–	–	++
17	42	35	–	–	–	++	–	–	–
18	41	43	–	–	–	++	–	–	–
19	37	89	–	–	–	++	–	–	–
20	30	82	–	–	–	++	–	–	–
21	34	11	–	–	–	–	–	++	–
22	30	8	–	–	–	+	–	–	++
23	32	16	–	–	–	++	–	–	–
24	16	7	–	–	–	++	–	–	++
25	18	38	–	–	–	++	–	–	–
26	4	8	–	–	–	+	–	+	–
27	4	30	–	–	–	++	–	–	–
28	2	61	–	–	–	++	++	++	++
Bark sample (gm)			10	10	10.5	13	10	10	10
Chromatogrammed sample (µl)			5	5	5	4	5	5	5

[a] 1. 2% acetic acid
2. n-Butanol-acetic acid-water (63:10:27)

– = Absent; + = <30 mm^2; ++ = 30 mm^2 to 200 mm^2; +++ = >200 mm^2.

Table 30. Mean hR_F-values with qualitative and semi-quantitative variation in components of American chestnut infected inner bark soluble in methonal as determined by spot size on two-dimensional silica gel thin-layer chromatograms

Spot No.	Mean hR_F-value in:[a] 1	2	Tree number 6	7	8
1	2	52	++	++	++
2	12	15	++	++	+
3	20	45	++	-	+
4	20	54	++	++	+
5	35	6	++	-	++
6	36	14	-	-	+
7	37	56	++	+++	+++
8	38	82	-	-	++
9	44	12	-	-	+
10	43	24	-	-	+
11	49	72	-	-	+
12	60	26	-	-	++
13	61	38	-	-	++
14	32	32	-	-	++
15	9	53	-	-	++
16	37	7	++	-	++
17	54	83	++	++	+
18	0	27	++	++	-
Bark sample (gm)			13	10	5.7
Chromatogrammed sample (µl)			4	5	5

[a] 1. 2% acetic acid
2. n-Butanol-acetic acid-water (63:10:27)

- = Absent; + = <30 mm^2; ++ = 30 mm^2 to 200 mm^2; +++ = >200 mm^2.

spot 9 and the appearance of a long, tailing spot (16) in the infected bark extract. Spray reagents yield little assistance in the identification of spot 16, but it hardly moves in the BAW solvent and could be either a polymer of some kind (tannin) or a glycoside.

No components of American chestnut root bark extracts soluble in methanol matched hR_F-values with components of any other sampling group extracted in this same solvent. However, six major component spots from twig extracts in methanol correspond to major spots from sound bark extracts in the same solvent (Table 28).

Relatively few components found in methanol-soluble extracts of exotic chestnut bark correspond with components found in American chestnut sound stem extracts. Spots 6, 10 and 11 of the exotic chestnut extracts correspond with 16, 9 and 5 of the American chestnut extracts, respectively.

Mean hR_F-values and qualitative and semi-quantitative data for individual trees sampled for American sound inner bark are presented in Table 29. A total of 28 component spots were located on thin-layer chromatograms of methanol-soluble extracts from seven individual tree samples of American chestnut sound stem inner bark. No single tree had all components, and the total number of spots obtained from a given single tree ranged from only 3 (tree 7) to 16 (tree 9). Trees 6, 7 and 8 did not have components 16 through 28 and initially it appeared that these components were those not extracted with anhydrous diethyl ether from trees 9 through 12. However, it must be noted that tree 10 exhibited only one (28) of the component spots 14 through 28, while 11 and 12 have only three and four component spots, respectively, of

the group 16 through 28. Therefore, it seems possible that these four trees could be entirely devoid of ether-soluble components.

Only components 3 and 7 appeared in every tree sampled. Component 3 was present in larger amount in tree 8 than in the other trees sampled, while component 7 was more plentiful in trees 9, 10 and 11 than in the others.

Mean hR_F-values with qualitative and semi-quantitative data are presented in Table 30, p. 95, for the American chestnut infected inner bark extracts. Tree number 8 is apparently very rich in components extractable in methanol; a 5.7-gram bark sample from this tree was extracted, compared with 10- and 13-gram samples from trees 7 and 6. In spite of this, tree 8 exhibited 17 extractable components compared with 6 and 9, respectively, for the other two. Samples from trees 6 and 7 had component 18 present in fair quantiy, while it was absent from tree 8. Only components 1, 2, 4, 7 and 17 were present in all samples. There was more of component 7 present in samples from trees 7 and 8 than from tree number 6, in spite of the fact that the bark sample from tree 6 was larger than from either of the other two trees.

Mean hR_F-values with qualitative and semi-quantitative data for American chestnut root bark extracts are presented in Table 31. Variation was great for this fraction; none of the components present in methanol-soluble extracts from trees 9 and 10 were present in extracts from tree 12. Their chromatographic patterns were completely different (Figure 18, p. 87). Components 1 through 8 were present in samples from tree 9, spots 1 through 5 appeared in samples from tree number 10, and components 9 through 16 were present in samples from tree 12

Table 31. Mean hR_F-values with qualitative and semi-quantitative variation in components of American chestnut root inner bark soluble in methanol as determined by spot size on two-dimensional silica gel thin-layer chromatograms

Spot No.	Mean hR_F-value in:[a] 1	2	Tree number 9	10	12
1	9	36	+++	++	-
2	12	58	+++	+++	-
3	12	70	+++	+++	-
4	14	42	-	++	-
5	12	16	++	+++	++
7	14	84	++	-	-
8	2	56	++	-	-
9	34	7	-	-	+++
10	26	15	-	-	++
11	19	39	-	-	++
12	48	40	-	-	+++
13	33	51	-	-	+
14	27	53	-	-	++
15	32	68	-	-	++
16	23	68	-	-	++
17	13	75	++	-	-
Bark sample (gm)			10	10	10
Chromatogrammed samples (µl)			5	5	5

[a] 1. 2% acetic acid
2. n-Butanol-acetic acid-water (63:10:27)

- = Absent; + = <30 mm^2; ++ = 30 mm^2 to 200 mm^2; +++ >200 mm^2.

(Figure 18, p. 87; Table 28, p. 93). Component 4, present in samples from tree number 10,was absent from tree number 9. Component 17, present in samples from tree number 9, was absent from tree number 10 samples. Quantitatively, more of component 1 was observed in samples from tree 9 than from tree 10 samples, while the opposite was true for component 5.

Qualitative and semi-quantitative variation among individual tree samples of American chestnut twigs is presented in Table 32 with mean hR_F-values for all spots in two solvents. Tree number 7 had every component (23) present in the methanol-soluble extract, while tree 10 exhibited only three component spots (3, 4 and 7); these three component spots were found in every tree sampled. Quantitative variation was, however, noted in components 3, 4 and 7, tree 10 having the lowest amount of these components. Tree number 7, which had the greatest total number of spots, had as much or more of all components than the other trees sampled, with the exception of spots 10, 12 and 21.

Qualitative and semi-quantitative data for the methanol-soluble fraction of exotic chestnut inner bark extractives are presented in Table 33, with mean hR_F-values for all component spots. No tree had all 12 spots present in the extracts; while number 21 (hybrid) lacked spots 1, 2, 5 and 7, trees 20 and 22 (C. mollissima) were missing only spot 9. Compounds 4, 6 and 12 varied in a quantitative manner. Tree 22 (C. mollissima) had less of component 4 than the other two trees sampled, tree 21 (hybrid) had a greater amount of component 6, and tree 22 exhibited more of component 12 than the other two trees sampled.

Table 32. Mean hR_F-values with qualitative and semi-quantitative variation in components of American chestnut debudded twigs soluble in methanol as determined by spot size on two-dimensional silica gel thin-layer chromatograms

Spot No.	Mean hR_F-value in:[a] 1	2	Tree number 7	9	10	11	12
1	10	14	++	+	-	+	+
2	15	13	++	+	-	+	-
3	18	38	++	++	+	++	++
4	23	39	+++	++	+	++	++
5	34	8	++	++	-	++	-
6	34	15	++	++	-	++	++
7	37	56	++	++	+	++	++
8	31	66	++	++	-	++	+
9	45	7	++	++	-	++	-
10	45	15	+	++	-	++	-
11	45	24	++	+	-	++	-
12	58	49	+	-	-	++	-
13	55	75	++	-	-	++	++
14	67	48	++	-	-	++	-
15	62	38	++	++	-	-	-
16	54	39	++	++	-	-	-
17	44	76	++	++	-	-	-
18	45	89	+	+	-	-	-
19	35	82	+	+	-	-	-
20	24	54	++	-	-	-	++
21	22	23	+	++	-	-	+
22	1	23	++	-	-	-	-
23	4	48	++	+	-	-	-
Bark samples (gm)			10	12.4	10	10	8
Chromatogrammed samples (µl)			5	5	5	5	5

[a] 1. 2% acetic acid
2. n-Butanol-acetic acid-water (63:10:27)

- = Absent; + = <30 mm^2; ++ = 30 mm^2 to 200 mm^2; +++ = >200 mm^2.

Table 33. Mean hR_F-values with qualitative and semi-quantitative variation in components of exotic chestnut sound inner bark soluble in methanol as determined by spot size on two-dimensional silica gel thin-layer chromatograms

Spot No.	Mean hR_F-value in:[a] 1	2	Tree number 20	21	22
1	9	14	+	-	+
2	22	22	+	-	+
3	23	21	++	++	++
4	32	69	++	++	+
5	36	18	+	-	+
6	56	42	++	+++	++
7	51	69	+	-	+
8	49	87	+	+	-
9	61	27	++	++	-
10	47	2	++	++	++
11	37	8	++	++	++
12	25	7	+	+	++
Bark samples (gm)			10	10	10
Chromatogrammed samples (µl)			5	5	5

[a] 1. 2% acetic acid
2. n-Butanol-acetic acid-water (63:10:27)

- = Absent; + = <30 mm^2; ++ = 30 mm^2 to 200 mm^2; +++ = >200 mm^2.

Bioassays

It would be desirable to develop a method by which small quantities of extract could be bioassayed for activity inhibitory to Endothia parasitica. Previous work (Barnett, 1967) demonstrated that water extracts from American chestnut inner bark used in a growth medium inhibited diameter growth of the fungus more than similar extracts from exotic chestnut trees. Since this result is not consistent with the relationship of resistance found in nature, it was felt that the need was for a rapid, simple test requiring little material in which many compounds or extracts could be tested for inhibitory activity relative to controls. By modifying a method described by Olsen (1971) such a technique was developed.

By allowing extract components to diffuse into agar seeded with spores of the fungus and directly comparing fungal growth on the treated agar with growth on an identical control area, inhibitory activity can be determined by subjecting data on relative colony size after a specific period of growth to analysis by the chi-square (X^2) test (Snedecor, 1957). Data are easily obtained and analyzed and, if taken after successively longer periods of time, allow comparison of relative inhibitory activity of treatments or extracts.

In these tests 50 µl of crude extract was added to circles on filter paper. Controls consisted of either 50 µl of the appropriate solvent or nothing, applied to a circle beside test circles. All circles were seeded with germinated pycnidiospores of the blight fungus in an identical manner. After varying time periods (depending upon room temperature, light, etc.) tests were scored, data arranged in contingency

tables and analyzed by the chi-square test. All tests were run two times, but no statistical difference was noted between tests of the same material. A statement of observed differences (**) indicates rejection of the null hypothesis at the 5 percent level of probability.

The method used for computing chi-squares will be demonstrated for the chloroform fraction. Discussion of the remaining fractions will include only relevant inhibition proportions and the chi-square values for specific comparisons.

Chloroform fraction bioassays were evaluated after two time periods (44 and 64 hours) so the relative inhibitory activity of different extracts could be compared. The X^2 data and analysis for the 44-hour observation is presented in Table 34.

Table 34. Relative growth of E. parasitica on chloroform-soluble extracts from inner bark of Castanea 44 hours after test initiation

Sample	Inhibited	Not Inhibited	Total	Proportion Inhibited
American 6-Sound	9	0	9	1.0
American 12-Sound	10	0	10	1.0
American 12-Root	9	1	10	0.90
American 6-Infected	9	1	10	0.90
Exotic 20	8	0	8	0.8889
Control	3	42	45	0.06667
Total	46	44	90	0.5111

$$X^2 = \frac{9(1.0) + 10(1.0) + 9(0.90) + 8(1.0) + 8(0.8889) + 3(0.06667) - 46(0.5111)}{(0.5111)(0.4889)}$$

$$= 71.6792^{**} \quad (df = 5)$$

Individual comparisons using 2 x 2 contingency tables were made as follows:

Sample	Exotic 20	Control	Total	American 12-Sound	American 12-Root	Total
Inhibited	8	3	11	10	9	19
Not inhibited	1	42	43	0	1	1
Total	9	45	54	10	10	20

X^2_c=26.395** X^2_c=0.0029 NS

American 12-Sound vs Exotic-20 X^2_c = 0.0 NS

American 6-Sound vs American 12-Sound X^2_c = 0.0 NS

American 6-Sound vs Control X^2_c = 32.593**

American 6-Infected vs. Exotic-20 X^2_c = 3.825 NS

American 6-Sound vs American 6-Infected X^2_c = 0.003 NS

American 12-Sound vs American 12-Root X^2_c = 0.0 NS

The X^2 values calculated for these and all 2 x 2 tables are corrected for continuity as described by Snedecor (1957) and by Steel and Torrie (1957).

From the first comparison it is apparent that all treatments inhibit fungal growth significantly more than controls, since the exotic material exhibited the smallest proportion of inhibition among all extracts tested. In this test controls were blank circles (no solvent). There was no significant difference between the inhibiton of extracts from sound stem and root inner bark of American chestnut tree number 12.

After 64 hours the proportions inhibited by the same test material were:

American 6-Sound	American 6-Infec.	American 12-Sound	American 12-Root	Exotic 20	Total
0.4444	1.0	0.50	0.0	0.25	0.2111

$X^2 = 38.562^{**}$ (df = 5)

Individual comparisons were made between some extracts with the following results:

Exotic-20 vs Control	$X^2_c = 5.820^{**}$
American 12-Sound vs American 12-Root	$X^2_c = 4.267^{**}$
American 6-Sound vs American 6-Infected	$X^2_c = 9.257^{**}$
American 12-Sound vs Exotic-20	$X^2_c = 0.354$ NS
American 6-Sound vs American 12-Sound	$X^2_c = 0.493$ NS
American 6-Sound vs Control	$X^2_c = 15.606^{**}$
American 6-Infected vs Control	$X^2_c = 45.884^{**}$
American 6-Infected vs Exotic-20	$X^2_c = 6.667^{**}$

Because an extract from exotic chestnut tree bark inhibited the fungus proportionately less than all other extracts being tested, the statistically significant result of comparing this extract with control indicates that all other extracts being tested also inhibited the fungus more than controls. Figure 21 is a photograph of comparative fungal growth rates on the chloroform-soluble extracts as tested by this method.

Comparisons among treatments disclosed no difference between the two American sound bark extracts, although sound bark extract from tree 12 was more inhibitory than root bark extract from the same tree. An extract from infected bark of American chestnut was the most inhibitory tested, suggesting that this species does form, in response to infection,

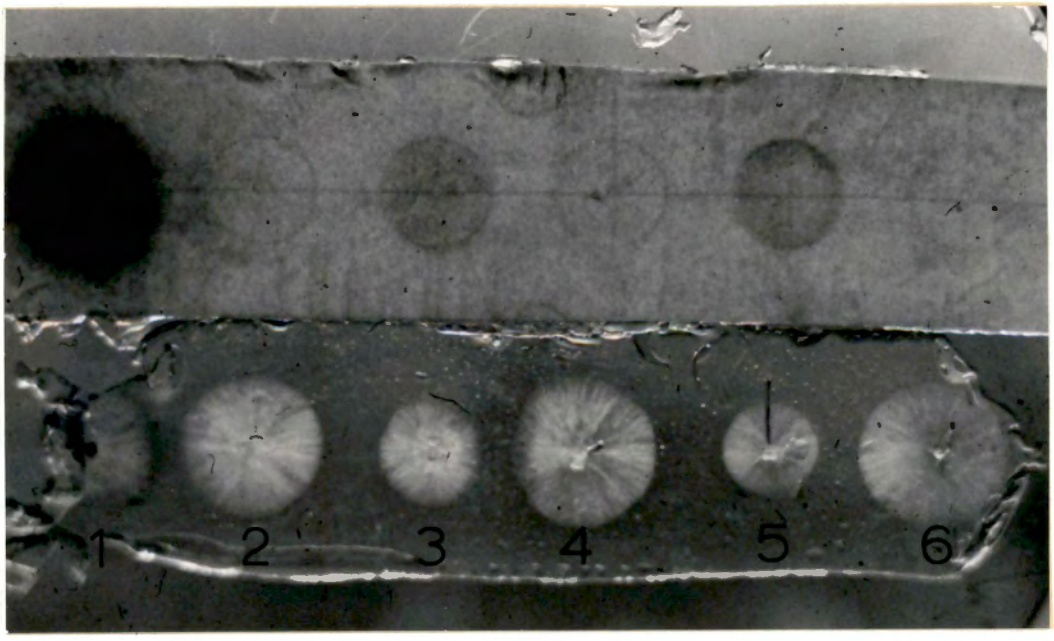


Figure 21. Comparative growth of E. parasitica in bioassay of chloroform-soluble extracts from inner bark of Castanea.

Upper strip-paper on which crude extracts were spotted; lower strip-colonies growing on agar removed from paper strip. Spots on paper (1, 3, 5) indicate relative depth of color of extracts; spots 2, 4, and 6 are controls. Spot 1 = American 12-sound; 3 = American 12-root; 5 = exotic 20. Photographed by transmitted light.

compounds inhibitory to the chestnut blight fungus *in vitro*.

Diethyl ether-soluble fraction bioassays were scored 51 hours after seeding. Proportions of each sample inhibitory to the fungus are as follows:

American 8-Inf.	American 8-Sound	American 10-Root	American 10-Sound	Hybrid 21	Control	Total
0.0	1.0	0.50	0.7778	0.70	0.2766	0.4468

$X^2 = 31.047^{**}$ (df = 5)

Individual comparisons yielded the following results:

American 10-Sound vs Control	$X^2_c = 7.631^{**}$
Hybrid-21 vs Control	$X^2_c = 4.764^{**}$
American 10-Root vs Control	$X^2_c = 1.011$ NS
American 10-Root vs American 10-Sound	$X^2_c = 0.6038$ NS
American 10-Root vs Hybrid-21	$X^2_c = 0.2083$ NS
American 10-Root vs American 8-Sound	$X^2_c = 6.667^{**}$
Hybrid-21 vs American 10-Sound	$X^2_c = 0.8211$ NS
Hybrid-21 vs American 8-Sound	$X^2_c = 3.5294$ NS

In this test, in contrast to the results of the chloroform fraction, extracts of infected bark from American chestnut trees did not inhibit growth of the fungus relative to controls. Similarly, an extract from root inner bark of American chestnut tree number 10 did not inhibit growth of the fungus relative to controls.

Comparisons between various extracts disclosed no difference in inhibitory action between extracts from roots and sound bark of American chestnut tree number 10. Neither of these extracts demonstrated differences from the hybrid chestnut extract, nor did the hybrid

chestnut bark extract differ from extract of sound bark of American chestnut tree number 8. Extracts from sound bark of American chestnut tree 8 were more inhibitory than an extract from roots of tree 10 of the same species.

Bioassays of acetone-soluble fractions were evaluated 45 hours after initiation. Proportions of each treatment showing inhibitions were:

American 8-Sound	American 8-Inf.	American 10-Sound	American 10-Root	Hybrid 21	Control	Total
0.60	0.90	1.0	0.70	0.90	0.160	0.490

$$X^2 = 47.899^{**} \text{ (df = 5)}$$

All extracts were significantly more inhibitory than controls. Results of individual comparisons were as follows:

American 8-Sound vs Control $X^2_c = 6.727^{**}$

American 8-Sound vs American 8-Infected $X^2_c = 4.267^{**}$

American 8-Sound vs Hybrid-21 $X^2_c = 4.267^{**}$

American 8-Sound vs American 10-Sound $X^2_c = 7.812^{**}$

American 10-Sound vs American 10-Root $X^2_c = 6.274^{**}$

American 10-Sound vs Hybrid-21 $X^2_c = 4.211^{**}$

Acetone-soluble extracts from American chestnut infected bark inhibited the fungus more than sound bark extracts from the same tree. However, extract from the hybrid chestnut tree inhibited fungal growth more than sound bark extract from this American chestnut (tree 8), but less than another American chestnut (tree 10). A comparison of components found in these two individual extracts (Table 22, p. 78) disclose that tree number 10 exhibits components 18, 22, 24 and 25 not possessed by tree

number 8, but lacks components 1 and 11 which are found in tree number 8. Some quantitative variation is also noted, since tree number 10 has less of components 12 and 19 but more of component 14. The inner bark extract from American chestnut tree 10, which was most inhibitory, was also more inhibitory than the root extract from this same tree.

Methanol-soluble fraction bioassays were evaluated 44 hours after initiation. Proportions inhibited were:

American 8-Sound	American 8-Infec.	American 10-Root	American 11-Sound	Hybrid 21	Control	Total
0.90	1.0	0.70	0.40	0.50	0.240	0.470

$X^2 = 31.674^{**}$ (df = 5)

The following individual comparisons were made:

American 11-Sound vs Control	$X^2_c = 0.4261$ NS
Hybrid-21 vs Control	$X^2_c = 1.642$ NS
American 10-Root vs Control	$X^2_c = 6.162^{**}$
American 8-Infected vs Control	$X^2_c = 17.584^{**}$
American 8-Sound vs Hybrid-21	$X^2_c = 5.952^{**}$
American 8-Sound vs American 11-Sound	$X^2_c = 3.516$ NS
American 8-Sound vs American 8-Infected	$X^2_c = 4.211^{**}$
American 11-Sound vs American 10-Root	$X^2_c = 3.232$ NS

As in some previous tests, extract from infected bark of American chestnut was more inhibitory than a sound bark extract from the same tree and more inhibitory than controls. Extracts from neither the American chestnut sound bark from tree number 11 nor from the hybrid individual were more inhibitory than controls. An extract from the roots of an American chestnut tree were no more inhibitory than stem bark extracts from the same species.

CHAPTER IV

DISCUSSION AND CONCLUSIONS

Although a total of 227 phenolic components were separated chromatographically from five samples extracted with four solvents in this study, none were found to be identical to one of the 15 standard compounds also chromatogrammed (Table 7, p. 42). These standards included simple phenols, phenolic acids and flavonoid glycosides with their corresponding aglycones. While some unknown components did co-chromatograph with standards, their color reactions did not correspond, thus they were not identified as being the same compound. Therefore, while no unknown phenolic compounds were specifically identified, some possibilities are eliminated.

An attempt was made to determine the character of phenols from American and exotic chestnut bark by using the ferric chloride reagent. This was not successful; in no case was the presence of catechol indicated (green color) (Hergert, 1960). Most components which reacted at all displayed a variety of colors ranging from bluish gray or faint lavender to dark blue or purple. A few spots turned faint to dark brown, suggesting a 3- or 5-hydroxy flavones (Geissman, 1955). The greatest proportion of components present in these extracts in quantity great enough to react with this reagent appear to be of the pyrogallol or phloroglucinol-resorcinol type. In comparing American sound stem (six components) and exotic bark extracts (five components) no real difference was observed in the total number of components reacting positively to this reagent. This result does not support Nienstaedt's

(1951; 1953) theory that the tannins of exotic chestnut are primarily of the pyrogallol-type while those of American chestnut are of the catechol-type. His extracts were, however, made with hot water, which might remove more tannin components than the stepwise extraction with organic solvents.

Perhaps the most noteworthy observation was the different chromatographic patterns exhibited by sound and infected stem inner bark samples from the same American chestnut trees. A total of twelve components from all four solvents were found to be indistinguishable, supporting work by Bazzigher (1955) who noted "decomposition" of tannins in parts of C. sativa infected by the blight fungus. This observation is significant when considered in connection with bioassay results. In all solubility groups except diethyl ether, crude extracts from American chestnut infected stem inner bark samples were more inhibitory to fungal growth than extracts of other samples. American chestnut trees infected by the blight fungus may, therefore, form compounds extractable with organic solvents which inhibit growth of the fungus in vitro.

Only nine components in three of the extract groups from American sound and exotic chestnut inner bark extracts (no phenolic compounds were found in diethyl ether extracts of exotic chestnuts) were found to be indistinguishable. Therefore, unless one or more of the seven common components are found to be inhibitory to the blight fungus there is little promise of using the more resistant C. mollissima as a chemical model for selection of resistant C. dentata individuals.

Significant differences were noted between individual American

chestnut sound stem inner bark extracts soluble in methanol in their inhibitory effect on the fungus in bioassays. Such variation can be used in a breeding program based upon selection of resistant individuals. Evidence presented here indicates that American chestnut sound stem bark extracts are more inhibitory than exotic chestnut extracts.

Nienstaedt (1953) presented evidence that the tannins of the more resistant species are more soluble than those from the less resistant species, at least when extracted with hot water. His organic solvent extracts yielded bioassay results similar to those presented here. Perhaps extraction with hot water only is capable of removing compounds from C. mollissima which are toxic to the blight fungus in vitro. The fact that Barnett (1967) did not confirm Nienstaedt's (1953) results while using hot water extracts adds to the confusion, however.

Crude sount root inner bark extracts from American chestnut individual trees were consistently less inhibitory in bioassay tests than sound stem extracts from the same trees. This observation implies that although stumps and roots of this species are high in tannin content, this factor, is not alone, responsible for lack of invasion of the root system by E. parasitica.

Many reactions with the various chromophoric reagents suggest the presence of flavonoid structures. This observation is of importance for blight resistance in American chestnut, since there is evidence that molecular size and shape have an effect on the ability of a phenolic compound to inactivate fungus enzymes; those based on the C_{15} monomer (such as β-catechin, L-epicatechin and leucoanthocyanins) have been found more effective than those based on a C_9 structure (such

as chlorogenic acid) (Byrde et al., 1960; Cole and Wood, 1961).

Significant to future selection and breeding is the presence of a fairly close correlation between acetone- and methanol-soluble extracts from American chestnut sound stem inner bark and corresponding extracts from debudded twigs from the same trees. Two spots found in acetone-soluble twig extracts correspond with significant components of sound bark extracts. One, tentatively identified as a guaiacol derivative, is absent from infected bark extracts and appears in twig extracts in the same relative amounts as in sound bark extracts. The other, present in lesser quantity in infected bark extracts than in sound bark extracts was tentatively identified as having the C_{15} structure common to the flavonoids; this compound was present in every twig extract in quantity approximating that found in sound stem bark extracts.

In comparing methanol-soluble extracts from American sound stem inner bark with twig extracts from the same trees, eleven component spots were found to be possibly identical. Four of these are rather prominent components which migrate to hR_F 35 to 50 in 2% acetic acid but move hardly at all in BAW. This characteristic, plus their reaction with color reagents, suggests glycosides of catechin or leucocyanidin, although their reaction with Pauly regent I indicates that they could be derivatives of guaiacol, vanillic acid or alpha-hydroxy guaiacol with phloroglucinol-resorcinol (meta) substitution. Another component present in twig extracts which matches the most prominent component in sound stem bark extracts, is also found in infected stem bark extracts.

Of particular interest, from the breeding standpoint, is the broad pattern of qualitative and quantitative variation noted among components from American chestnut trees sampled for sound stem bark. Although chloroform- and ether-soluble extracts were not evaluated quantitatively, because of generally small spot sizes, the pattern of qualitative variation is significant, particularly in the case of chloroform extracts. Of ten components found in this group, only three were present in every tree and four were found in only one tree.

A total of 22 components were noted in acetone-soluble extracts from American chestnut sound stem inner bark. Only nine were present in every tree and of these only one varied quantitatively. Of the remaining 13 components not observed in every tree, eight varied in a quantitative manner, suggesting that these components could be present in the other trees, but in too small quantities to be detected by the method employed.

Variation was greatest in the methanol-soluble extractives from American chestnut sound stem inner bark (Table 29, p. 94). Of a total of 28 components, only two were present in every tree sampled, and quantitative variation was noted in both of these. The smallest number of components found in extracts from a given tree was three and the largest number was 16; many components were noted in only one or two trees.

For meaningful quantitative studies it will be necessary to use a more accurate chromatographic technique, such as gas-liquid chromatography, but the data obtained in this work has provided evidence of

sufficient variation to encourage the continuance of a selection program for breeding American chestnut trees with resistance to the blight fungus, Endothia parasitica.

CHAPTER V

SUMMARY AND RECOMMENDATIONS

Stepwise Soxhlet extraction of inner bark samples from American chestnut sound stem, infected stem and sound root inner bark and debudded twigs as well as exotic chestnut inner bark was carried out with four organic solvents of progressively increasing polarity. Components of these crude extracts were separated on silica gel thin layers to study comparative phenolic composition. Qualitative and quanitative variation among samples of the same type, extracted with the same solvents were investigated. Extracts were also bioassayed to test them for inhibitory action toward the chestnut blight fungus, Endothia parasitica. Component spots were determined to be phenolic by their blue color reaction with ferric chloride-potassium ferricyanide reagent. Use of a series of more selective reagents permitted some components to be tentatively assigned to more specific chemical groups.

While the objectives of this study, as originally outlined, were not completely met, significant progress was made in each of the areas. Mean hR_F-values and color reactions of all spots found in thin-layer chromatograms of extracts from both American and exotic chestnut species disclose considerable difference in the phenolic constitution; only nine components extracted with three organic solvents were found to most likely be the same compounds. While more qualitative and quanitative variation was noted among individual American chestnut trees than among individual exotic trees, this is probably a reflection of the fact that two of the three exotic chestnut trees sampled were full-sib progenies. No determination was made concerning the effect of these differences upon blight resistance.

While some components were shown to be common to both the American and exotic species of chestnut, none of these compounds were specifically identified. Some were, however, placed in a broad chemical class.

It was shown that considerable qualitative and quantitative variation does exist among American chestnut individual trees. Since no specific compound was shown to be directly connected with blight resistance, it is impossible to make a statement concerning the possibility of multigenic control of this factor. Inner bark components extracted with organic solvents did inhibit E. parasitica in vitro. The inhibition noted was not, however, correlated with known resistance of the species, i.e,, extracts from American chestnut inner bark was more inhibitory than similar extracts from exotic (blight resistant) chestnut individuals. The most inhibitory extract bioassayed came from infected American chestnut inner bark.

Other results of this work indicate that:

1. As disclosed by the ferric chloride reagent, there is no discernible difference between American and exotic chestnut sound inner bark extracts soluble in organic solvents relative to the class of phenols present.
2. There appears to be a "decomposition" of tannin or polyphenol components of American chestnut sound stem inner bark in areas infected by the blight fungus. There are formed, by American chestnut trees infected by the blight fungus, extractable compounds which inhibit growth of E. parasiticas in vitro, relative to controls and sound stem bark extracts from the same trees.

3. There are considerable differences between American chestnut sound stem inner bark extract components and exotic chestnut extracts of the same type. Only nine components found in extracts of three organic solvents were observed as being indistinguishable.
4. There is little correlation between sound root and sound stem inner bark components from American chestnut individual trees.
5. In bioassays American chestnut sound root inner bark extracts were consistently less inhibitory than the corresponding sound stem inner bark extracts.
6. Colors obtained with chromophoric reagents indicate extractable components from inner bark of C. dentata, C. mollissima, and a C. mollissima x C. alabamensis hybrid consist primarily of derivatives of flavonoids, catechins, leucoanthocyanins, guaiacol or vanillic acid.
7. Since there is a fairly close correlation between twig and sound inner bark extracts of individual C. dentata trees, it may be possible to select American chestnut trees resistant to the blight based on chromatographic composition of twig extracts, once components common to both sample groups can be shown to be of significance in a blight resistant reaction, either directly or indirectly.

Results obtained in this study indicate that three specific studies would most likely contribute to knowledge about various extracts with a minimum expenditure of time and money:

1. Since the chloroform-soluble fraction of the American infected bark extract was the most inhibitory of those tested in bioassays, it should be investigated to determine what component or components are responsible for this factor. Since it is a relatively simple extract, having only a few components, this analysis should be done by using column chromatography in conjunction with infrared and mass spectroscopy. Further information concerning the specific inhibitor involved might be obtained by the method described by Olsen (1971).
2. The noted variation in inhibition of the blight fungus *in vitro* by American chestnut inner bark extracts should be investigated further. By attempting to correlate results of bioassays on bark extracts from individual American chestnut trees with the qualitative variation observed, some idea of the compound or compounds responsible for the noted inhibition might be obtained. Such a study should include extracts of infected bark from both American and exotic chestnut individuals so comparative inhibition of extracts from the two infected barks can be directly compared.
3. The noted differences in chemical composition of sound and infected American chestnut bark extracts should encourage further research. Hydrolysis (with acid and/or enzymes) and oxidation of aliquots of sound bark extracts followed by chromatography and subsequent comparison with chromatograms of infected bark extracts may provide information

concerning the action of the fungus enzyme system upon the chemical constituents of American chestnut inner bark.

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APPENDIX

Preparation of nutrient medium and single-spore cultures of Endothia parasitica for use in bioassaying chestnut extracts for activity against the fungus

The potato-dextrose agar (PDA) of Riker and Riker (1936) was diluted and still supported growth of the fungus. The following preparation was finally selected for use in all bioassays:

Agar	14 gm
Potatoes (peeled and sliced)	100 gm
Dextrose	10 gm
Water	1,000 ml

Potatoes are cooked in one-half the water for 30 min. in the autoclave. Agar is melted into the other one-half water. Potato juice is then decanted into the melted agar and the volume adjusted to 1,000 ml with water. The dextrose was then added.

Single spore cultures growing on a medium identical to the one used in the bioassay were prepared as follows:

One-half a 15 cm colony (20-30 days old) of Endothia parasitica was placed in a Waring blender with 500 ml autoclaved, distilled water. After macerating in the blender for one minute, 50 ml of spore suspension was collected by straining through a double layer of Kleenex tissue into a graduated cylinder. Collected spores were added to 950 ml autoclaved, distilled water and shaken well. One milliliter of this suspension was in turn added to 100 ml water to make the stock spore suspension. Fifteen centimeter petri dishes containing a 1-2 mm thick layer of the PDA medium described above were inoculated with 1 ml of stock suspension and shaken well to distribute the spores evenly over the plate.

Table 35. Mean hR_F-values and the interval included by ± two standard errors for phenolic component spots of American chestnut sound inner bark extracts soluble in acetone, chromatogrammed on silica gel thin layers

Spot No.	No. Samples	Solvent 1[a]		Solvent 2[a]	
		Mean hR_F	Interval	Mean hR_F	Interval
1	4	7.2	4.2-10.2	39.2	36.4-42.0
2	7	10.3	7.5-13.1	68.0	66.0-70.0
3	8	9.0	8.3- 9.7	7.8	7.3- 8.3
5	8	18.8	16.9-20.7	53.5	52.1-54.9
6	8	30.8	27.8-33.8	45.4	14.2-46.6
7	8	31.9	29.1-34.7	69.1	66.9-71.3
8	8	39.4	37.2-41.6	29.1	28.1-30.1
9	8	45.6	43.4-47.8	46.4	45.1-47.7
11	2	43.5	38.5-48.5	20.5	19.5-21.5
12	7	41.8	38.2-45.4	55.4	53.2-58.7
13	5	46.4	42.6-50.2	72.6	70.0-75.2
14	8	51.2	48.9-54.0	53.7	51.9-55.5
15	4	48.5	46.6-50.4	60.5	59.2-61.8
16	5	52.0	49.0-55.0	73.6	70.8-76.4
17	8	57.0	54.6-59.4	72.5	70.1-74.9
18	5	47.2	42.8-51.6	85.2	82.4-88.0
19	7	30.0	28.1-31.9	18.8	17.8-19.8
20	4	14.5	13.2-15.8	46.0	43.4-48.6
22	2	18.0	16.0-20.0	27.5	26.5-28.5
24	3	25.0	22.0-28.0	46.3	43.9-48.7
25	3	31.7	24.3-39.1	50.7	48.1-53.3

[a] 1. Ethyl acetate-methanol-water (100:16.5:13.5)
2. n-Butanol-acetic acid-water (63:10:27)

Table 36. Mean hR_F-values and the interval included by ± two standard errors for phenolic component spots of American chestnut infected stem inner bark extracts soluble in acetone, chromatogrammed on silica gel thin layers.

Spot No.	No. Samples	Solvent 1[a]		Solvent 2	
		Mean hR_F	Interval	Mean hR_F	Interval
1	4	0	--	--	--
2a	4	5	4.2- 6.8	8	7.2- 9.2
2b	4	16	14.4-17.6	8	7.2- 9.2
3	2	11	10.5-12.5	24	23.5-25.5
4	3	22	19.0-25.0	1	0- 1.4
5	4	23	21.3-25.1	33	29.6-36.4
6	3	22	21.0-23.6	57	46.9-55.7
8	3	30	25.8-34.2	18	15.0-21.0
9	2	32	30.5-32.5	44	42.0-46.0
10	4	35	30.2-39.4	69	66.8-70.8
11	2	38	36.5-38.5	46	43.5-49.5
12	3	40	36.9-42.5	22	17.7-25.7
13	3	41	38.0-44.0	49	46.9-50.5
14	3	45	41.0-49.0	32	28.9-34.5
15	4	50	47.6-52.4	47	41.8-52.2
17	3	45	41.5-49.1	73	68.8-77.2
18	2	57	51.0-63.0	53	51.0-55.0
20	4	60	57.9-61.7	72	70.1-73.5

[a]1. Ethyl acetate-methanol-water (100:16.5:13.5)
2. n-Butanol-acetic acid-water (63:10:27)

Table 37. Mean hR_F-values and the interval included by ± two standard errors for phenolic component spots of American chestnut root inner bark extracts soluble in acetone, chromatogrammed in silica gel thin layers

Spot No.	No. Samples	Solvent 1[a]		Solvent 2	
		Mean hR_F	Interval	Mean hR_F	Interval
1	4	4	3.2- 4.8	7	6.2- 7.8
2	4	14	12.8-16.2	9	8.2- 9.8
3	4	22	20.4-23.6	32	30.8-33.2
4	4	27	25.9-28.5	15	13.3-17.1
5	3	32	32.0-33.4	58	55.0-61.0
6	4	37	34.6-39.0	47	45.5-48.9
7	4	44	41.8-46.2	56	53.6-57.4
8	4	46	45.2-47.8	44	41.8-46.2
9	3	20	19.0-20.4	18	14.0-22.0
10	2	27	25.0-29.0	1	0- 1.5

[a] 1. Ethyl acetate-methanol-water (100:16.5:13.5)
2. n-Butanol-acetic acid-water (63:10:27)

Table 38. Mean hR_F-values and the interval included by ± two standard errors for phenolic component spots of American chestnut debudded twig extracts soluble in acetone, chromatogrammed on silica gel thin layers

Spot No.	No. Samples	Solution 1[a]		Solution 2	
		Mean hR_F	Interval	Mean hR_F	Interval
1	4	3	2.3- 3.3	6	5.3- 6.3
2	4	9	7.8-10.2	8	7.2- 8.8
3	4	21	18.6-23.8	30	27.6-32.4
4	4	23	21.6-24.0	53	49.6-56.0
5	2	27	23.0-31.0	16	16
6	2	34	30.5-36.5	50	46.5-52.5
7	3	41	37.8-45.2	47	47
8	4	46	43.1-49.9	55	52.4-57.2
9	4	48	45.4-50.2	76	73.2-78.4
10	4	49	45.6-52.0	42	38.6-45.0
11	4	52	49.0-55.0	84	80.8-86.8
12	3	57	54.3-59.1	89	88.6-90.0
13	4	60	56.1-62.9	73	70.4-75.6

[a] 1. Ethyl acetate-methanol-water (100:16.5:13.5)
2. n-Butanol-acetic acid-water (63:10:27)

Table 39. Mean hR_F-values and the interval included by ± two standard errors for phenolic component spots of exotic chestnut sound inner bark extracts soluble in acetone, chromatogrammed on silica gel thin layers

Spot No.	No. Samples	Solution 1[a]		Solution 2	
		Mean hR_F	Interval	Mean hR_F	Interval
1	4	1.8	0.3- 3.3	5.5	4.9- 6.1
2	4	9.2	7.9-10.5	9.2	8.2-10.2
3	2	14.0	12.0-16.0	49.0	49.0
4	4	18.8	17.1-20.5	31.8	30.8-32.8
5	2	26.0	26.0	61.5	58.5-64.5
6	4	36.8	36.3-37.2	68.0	65.8-70.2
7	4	37.5	36.5-38.5	48.5	46.5-50.5
8	2	39.5	36.5-42.5	65.5	64.5-66.5
9	4	44.8	43.3-46.3	57.5	55.8-59.2
10	4	50.0	48.8-51.2	83.8	82.3-85.3
11	4	42.5	41.2-43.8	73.0	71.2-74.8
12	2	37.0	35.0-39.0	60.0	58.0-62.0
13	1	37	37	55	55
14	2	22.0	22	48	48
15	2	14.5	11.5-17.5	35.5	34.5-36.5
16	1	58	58	90	90

[a]1. Ethyl acetate-methanol-water (100:16.5:13.5)
2. n-Butanol-acetic acid-water (63:10:27)

Table 40. Mean hR_F-values and the interval included by ± two standard errors for phenolic component spots of American chestnut sound stem inner bark extracts soluble in methanol, chromatogrammed on silica gel thin layers

Spot No.	No. Samples	Solvent 1[a]		Solvent 2	
		Mean hR_F	Interval	Mean hR_F	Interval
1	3	2	0.8- 3.2	23	20.7-25.9
2	2	2	0.5- 2.5	38	37.5-39.5
3	8	14	12.4-15.8	15	14.0-16.0
4	2	19	19	58	54.0-62.0
5	6	37	36.0-38.3	7	6.2- 8.2
6	3	38	32.5-42.9	16	14.8-17.2
7	8	37	33.8-39.8	57	55.5-58.7
8	7	42	39.2-44.8	84	82.2-85.8
9	3	49	46.3-51.1	5	2.3- 7.1
10	2	51	47.0-55.0	13	7.0-19.0
11	1	52	52	25	25
12	1	52	52	75	75
13	5	63	56.6-69.0	34	31.2-36.4
14	2	60	53.5-67.5	40	36.0-44.0
15	4	1	0.0- 3.0	5	1.2- 8.8
16	2	51	49.0-53.0	40	34.0-46.0
17	1	42	42	35	35
18	1	41	41	43	43
19	1	37	37	89	89
20	1	30	30	82	82
21	1	34	34	11	11
22	2	30	28.5-30.5	8	8
23	1	32	32	16	16
24	3	16	13.6-19.4	7	6.1- 7.3
25	1	18	18	38	38
26	2	5	3.5- 5.5	8	4.5-10.5
27	1	4	4	30	30
28	4	2	0- 3.8	61	56.6-65.0

[a] 1. 2% acetic acid
2. n-Butanol-acetic acid-water (63:10:27)

Table 41. Mean hR_F-values and the interval included by ± two standard errors for phenolic component spots of American chestnut infected stem inner bark extracts soluble in methanol, chromatogrammed on silica gel thin layers

Spot No.	No. Samples	Solvent 1[a]		Solvent 2	
		Mean hR_F	Interval	Mean hR_F	Interval
1	4	2	2.0	52	45.1-59.9
2	4	12	9.6-14.8	15	13.8-16.6
3	3	20	17.3-22.1	45	41.9-47.5
4	4	20	18.7-22.3	54	51.8-56.2
5	2	35	33.0-37.0	6	4.5- 6.5
6	1	36	36	14	14
7	4	37	35.0-39.0	56	54.0-58.4
8	2	38	34.5-40.5	82	80.5-82.5
9	2	44	42.0-46.0	12	4.5-18.5
10	1	43	43	24	24
11	1	49	49	72	72
12	2	60	50.5-68.5	26	20.0-32.0
13	2	61	59.0-63.0	38	36.0-40.0
14	2	32	20.5-42.5	32	32
15	1	9	9	53	53
16	1	37	37	7	7
17	3	54	50.5-58.1	83	76.5-90.1
18	1	0	0.0	27	27

[a]1. 2% acetic acid
2. n-Butanol-acetic acid-water (63:10:27)

Table 42. Mean hR_F-values and the interval included by ± two standard errors for phenolic component spots of American chestnut root inner bark extracts soluble in methanol, chromatogrammed on silica gel thin layers

Spot No.	No. Samples	Solvent 1[a]		Solvent 2	
		Mean hR_F	Interval	Mean hR_F	Interval
1	3	9	5.7-12.9	36	35.0-37.6
2	3	12	9.0-14.1	58	51.7-63.7
3	3	12	7.3-16.1	70	62.8-77.2
4	2	14	10.0-18.0	42	34.0-50.0
5	3	12	9.2-17.4	16	13.8-18.3
7	1	14	14	84	84
8	1	2	2	56	56
9	1	34	34	7	7
10	1	26	26	15	15
11	1	19	19	39	39
12	1	48	48	40	40
13	1	33	33	51	51
14	1	27	27	53	53
15	1	32	32	68	68
16	1	23	23	68	68
17	1	13	13	75	75

[a] 1. 2% acetic acid
2. n-Butanol-acetic acid-water (63:10:27)

Table 43. Mean hR_F-values and the interval included by ± standard errors for phenolic component spots of American chestnut debudded twigs soluble in methanol, chromatogrammed on silica gel thin layers

Spot No.	No. Samples	Solvent 1[a]		Solvent 2	
		Mean hR_F	Interval	Mean hR_F	Interval
1	5	10	8.3-12.1	14	13.2-15.6
2	4	15	13.9-16.1	13	12.2-13.8
3	5	18	16.8-18.8	38	36.0-40.4
4	5	23	21.2-24.8	39	37.2-40.0
5	4	34	32.0-36.4	8	7.0- 8.0
6	5	34	32.0-36.8	15	14.9-15.9
7	5	37	35.1-38.5	56	51.8-60.2
8	5	31	29.3-33.7	66	60.8-70.8
9	4	45	42.4-48.0	7	3.9- 9.1
10	4	45	42.0-48.0	15	11.0-19.0
11	4	45	41.6-48.4	24	22.2-26.2
12	4	58	57.1-58.3	49	43.8-54.2
13	3	55	50.7-59.9	75	66.4-83.6
14	3	67	67	48	43.8-52.2
15	2	62	50.0-74.0	38	32.5-42.5
16	2	54	44.0-64.0	39	31.0-47.0
17	2	44	42.0-46.0	76	73.5-79.5
18	2	45	39.0-51.0	89	85.0-93.0
19	2	35	33.0-37.0	82	76.0-88.0
20	2	24	20.5-26.5	54	52.5-54.5
21	3	22	18.7-25.9	23	20.9-25.7
22	1	1	1.0	23	23
23	2	4	0- 9.5	48	45.5-51.5

[a] 1. 2% acetic acid
2. n-Butanol-acetic acid-water (63:10:27)

Table 44. Mean hR_F-values and the interval included by two standard errors for phenolic component spots of exotic chestnut sound stem inner bark extracts soluble in methanol, chromatogrammed on silica gel thin layers

Spot No.	No. Samples	Solvent 1[a]		Solvent 2	
		Mean hR_F	Interval	Mean hR_F	Interval
1	2	9	7.0-11.0	14	8.5-11.5
2	2	22	18.0-26.0	22	19.5-25.5
3	5	23	18.6-26.6	41	39.1-42.5
4	4	32	28.0-36.4	69	67.6-70.4
5	2	36	24.0-48.0	18	17.5-19.5
6	4	56	52.8-58.8	42	39.6-43.4
7	2	51	45.0-57.0	69	61.0-77.0
8	3	49	46.0-52.0	87	84.0-90.0
9	3	61	58.7-63.9	27	24.5-30.1
10	3	47	44.0-50.0	3	1.4- 4.0
11	3	37	35.8-38.2	8	8.0
12	3	25	22.8-26.4	7	6.1- 7.3

[a] 1. 2% acetic acid
2. n-Butanol-acetic acid-water (63:10:27)

Table 45. Color reactions of phenolic component spots found on silica gel thin-layer chromatograms of acetone-soluble extracts from American chestnut sound stem inner bark

Spot No.	Visible light	UV 254 nm	UV 360 nm	NH_3 vapor visible light	NH_3 vapor 360 nm UV light	2% $FeCl_3$	Tet. Benzidine	Pauly I	Pauly I + II	Diaz. 4-nitro-analine	Neu's + 370 nm UV
1	-	-	-	-	-	-	-	vf Y	f Bn	-	-
2	-	f Gn	-	-	Gn fl	-	1 Bn	vf Y	Or	-	-
3	-	f B	-	-	B	d Bl	Bn	Or-Y	d Bn	Or-Bn	-
4	vf Bn	-	-	Gn fl	-	-	vf Y	vf Y	vf Y	-	-
5	-	-	-	-	Pu	-	-	-	1 R	-	-
6	-	-	abs	f Bn	Pu	d Bl	Bn	1 Y	Bn	Or	Bl fl
7	-	-	-	-	-	-	1 Bn	vf Y	Or	-	-
8	-	-	-	-	Pu	Bl-Gy	Or	Bn-Y	Or	Or	-
9	-	f B	-	f Bn	Pu	d Bl	Bn	Or-Y	1 Or	Or	Bl fl
10	-	-	abs	f Bn	-	-	vd R	-	-	1 Or	Pu
11	-	-	-	-	-	-	-	-	1 Or	Or	-
12	-	-	-	-	-	-	vd R	-	-	1 Or	-
13	-	-	-	vf Bn	-	Bl-Gy	vd R	Bn-Y	Or	Or	-
14	-	-	-	-	-	-	vd R	-	-	Or	-
15	-	-	-	-	-	-	-	vf Or	1 Bn	-	-
16	-	-	-	vf Bn	-	-	vd R	Bn-Y	Or	Or	-
17	-	-	-	vf Bn	-	Bl-Gy	vd R	Bn-Y	Or	Or	-
18	-	-	-	-	-	-	-	-	Or-R	Or-R	-
19	-	-	-	-	-	-	1 Or	-	1 Or	Or-Y	-
20	-	-	-	-	-	-	-	-	vf R	-	-
21	-	-	-	-	-	-	1 Bn	-	-	-	-

Abs = absorbs; d = dark; f = faint; fl = fluorescent; l = light; v = very.
B = Black; Bn = Brown; Bl = Blue; Gn = Green; Gy = Gray; Or = Orange; Pu = Purple; R = Red; Y = Yellow.

Table 46. Color reaction of phenolic component spots found on silica gel thin-layer chromatograms of acetone-soluble extracts from exotic chestnut sound inner bark

Spot No.	Visible light	UV 254 nm	UV 360 nm	NH_3 vapor visible light	NH_3 vapor 360 nm UV light	2% $FeCl_3$	Tet. Benzidine	Pauly I	Pauly I + II	Diaz. 4-nitro-analine	Neu's + 360 nm UV
1	Gn-Y	abs	abs	-	-	d Bl	1 Bn	1 Bn	1 Bn	Gn-Y	-
2	Gn-Y	abs	abs	-	-	d Bl	1 Bn	1 Bn	1 Bn	Gn-Y	-
3	-	f Gn	-	-	-	-	-	-	Pk	-	-
4	-	abs	-	-	-	1 Bl	Or	Or	Y-Or	Or	-
5	-	-	-	-	-	-	-	-	vf Pk	-	-
6	-	-	-	-	-	-	-	f Pk	Pk	-	-
7	Gn-Y	abs	abs	-	-	d Bl	Bn	Or	1 Bn	Gn-Y	-
8	-	abs	abs	-	-	-	f Bn	1 Pk	Pk	1 Gn-Y	-
9	-	-	-	-	-	1 Bl	Or	1 Pk	1 Pk	Gn-Y	-
10	-	-	abs	-	-	-	1 Bn	1 Pk	Pk	-	-
11	-	-	-	-	-	-	-	-	f Pk	-	-
12	-	-	-	-	-	-	-	-	f Pk	-	-
13	-	-	-	-	-	-	-	-	Pk	-	-
14	-	-	-	-	-	-	-	-	f Pk	-	-
15	-	-	-	-	-	-	-	-	Pk	-	-
16	-	-	-	-	-	-	-	-	Pk	-	-

abs = absorbs; d = dark; f = faint; l = light; v = very.
Bl = Blue; Bn = Brown; Gn = Green; Or = Orange; Pk = Pink; Y = Yellow.

VITA

Paul Edward Barnett was born at Jerryville (Webster County), West Virginia on March 19, 1936. He attended elementary schools in Cowen, West Virginia, and was graduated from Cowen High School in 1954. In June, 1954, he enlisted in the U.S. Air Force and served as an aerial and ground photographer in Massachusetts and Alaska. In September, 1959, he enrolled at Lees Junior College, Jackson, Kentucky, transferring to West Virginia University, Morgantown, West Virginia, in September, 1960. He was graduated from that institution with a Bachelor of Science degree in Forest Management in June, 1964, immediately accepting a research assistantship with the Forestry Department, The University of Tennessee. In June, 1967, he received a Master's degree with a major in Plant Physiology and continued study toward the doctorate as a graduate research assistant. In July, 1969, he was employed as a Botanist by the Tennessee Valley Authority, Division of Forestry, Fisheries and Wildlife Development, and is presently in that position.

He received the Doctor of Philosophy degree with a major in Agricultural Plant and Soil Science in December, 1972. He is a member of Xi Sigma Pi, Sigma Xi and The Society of American Foresters.

He is married to the former Eva Lynn Hammon of Webster Springs, West Virginia. They have two children, Cheryl Lynn and Orvan Andrew.