PHYTOTOXIC ACTION OF ENDOCONIDIOPHORA FAGACEARUM BRETZ

by

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INTRODUCTION

Vascular diseases of plants are, at the present time, the subject of intensive physiological and biochemical studies. The interest shown in these diseases does not stem from economic considerations alone, for fundamental to the ultimate goals of pathology is the urgent requirement for a more basic knowledge of parasitism and its causes. In this respect wilt diseases are unusual. They offer to the pathologist an opportunity to investigate a distinct pathological relationship unhampered by the complexities attendant upon the more intricate parasitic interactions common to the majority of plant diseases.

Endoconidiophora fagacearum Bretz, the causal organism of oak wilt, in common with other wilt pathogens, is confined until death of the host to the large non-living vessels of the xylem. Symptom expression generally arises as a result of such an association. In explanation of this, a tenable theory presupposes the existence of specific toxins released in the xylem by parasite or host and transported passively to a site of action in the living cell. On the basis of present evidence, however, the possibility also exists that symptoms arise as a result of secondary effects less directly linked to the metabolic activity of the pathogen.

It was the basic intent of this study to clarify and

characterize the mechanism of symptom development in oak wilt in relation to these two theories.

REVIEW OF LITERATURE

Tyloses and Their Role in Wilt Diseases

Basic studies on host parasite interactions in the oak wilt disease have centered around the extensive formation of tyloses in xylem vessels of diseased oak (Beckman *et al.* 1953b). Although a pronounced symptom of the wilt disease, tyloses are found in most species of the genus, *Quercus* (Williams 1942), arising from other causes such as drought, wounding, or senesence. Therefore, they are by no means diagnostic for this disease. Tylosis formation has also been observed as a widespread physiological phenomenon in many families of plants (Chattaway 1949; Chrysler 1908; Gerry 1914).

Arising as a consequence of vascular infection, tyloses have been found in watermelon (Sleeth 1933), grape (Esau 1949), tobacco (Powers 1954) and other plants (Clinton and McCormick 1936; van der Meer 1926). However, their occurrence in certain vascular diseases is probably not pronounced enough to account for wilting except in the case of oak wilt. In this disease tylosis formation is so extensive and rapid it is assumed to be the primary cause of wilting (Beckman *et al.* 1953b).

While tyloses are known to be hypertrophic proliferations through the pit adjoining parenchymatous ray cells with

vessel segments or tracheids (Esau 1949; Küster 1925), evidence as to their fundamental cause is fragmentary. According to Haberlandt (1921), tyloses arise in response to secretion of wound hormones by living cells. Isolation of a wound hormone-like substance from dried bean pods (Haberlandt 1921) and its subsequent identification as traumatic acid (English et al. 1939) have lent support to this theory. This acid has never been tested as a tylosis inducing agent. The restricted number of plants in which it induces any healing response, however, has cast serious doubt on the validity of its interpretation as a wound hormone or growth regulator (Bonner and English 1938; Davis 1949).

Exposure to oxygen as a result of wounding has also been proposed as a cause of tylosis formation (Klein 1923).

The former hypothesis appears to be more widely accepted although naturally occurring wound hormones, other than traumatic acid, have neither been characterized nor definitely established (Block 1952).

Evidence of the part played by pathogens in inciting tylosis formation is indirect. Using radio-active rubidium, the close correlation that exists in diseased red oaks between infection, incipient wilting, decline in the rate of water movement and formation of tyloses was demonstrated (Beckman et al. 1953b). As a consequence of this work and anatomical studies (Struckmyer et al. 1954), tyloses were

postulated to be the major cause of vascular plugging in diseased red oak. It was also suggested that they could arise through action of specific substances produced by E. fagacearum. More recent investigations have demonstrated the existence of growth stimulants in culture filtrates of this organism (Fergus and Wharton 1957).

Evidence that tyloses arise through stimulation by metabolic products of the host has also been presented (Powers 1954). Tobacco plants infected with Phytophthora parasitici Dast. form tyloses but the response was initiated not by the parasite, but by products of host cells as a result of infection or wounding.

While the ability of several compounds to stimulate wound healing has been investigated (Brown and Cormack 1937; Davis 1949; LaRue 1941), apparently there has been no investigation of their effect on tylosis formation, although the latter is a wound response and a common growth phenomonon. The theory that accumulation of SH-containing compounds is positively correlated with wound healing responses (Hammet and Chapman 1938), has been partly confirmed (Davis 1949). Cysteine hydrochloride and glutathione, both sulfhydryl compounds, were found to stimulate wound healing in several species of plants. These appear to be of more universal application in this regard than any of the more specific growth regulators.

Wilt Disease Toxins

While toxins, or vivotoxins (Dimond and Waggoner 1953a) have long been implicated in the wilt disease syndrome (Hutchinson 1913), unequivocal evidence of their existence *in vivo* had not been established until recently (Dimond and Waggoner 1953b; Gäumann 1957). Since the last comprehensive review of wilt induction (Dimond 1955), a wide variety of metabolic products have been implicated, particularly with reference to tomato wilt (Gäumann 1957). Formation and function of some of these substances are well understood (Dimond and Waggoner 1953b; Gäumann 1957). Many other compounds, fusarinic acid (Yabuta *et al.* 1934), lycomarasmin (Gäumann 1951), and lateritin I (Gäumann *et al.* 1947; Cook *et al.* 1947) are less perfectly known. Through these discoveries the postulated mechanism of action of toxins in wilt diseases has been modified to incorporate interactions of all these compounds in the wilt syndrome (Gäumann 1957). The solution to this problem has therefore become infinitely more complex.

In addition to certain chemically characterized compounds which induce various aspects of the wilt disease, higher molecular weight substances such as fungus-synthesized polysaccharides (Hodgson *et al.* 1949) and gums (Bewley 1922; Rosen 1926; Struckmyer *et al.* 1954) have been implicated as physical agents in occlusion of vessel segments. Enzymes such as pectin methyl esterase induce vascular browning

(Gothoskar et al. 1953; Winstead and Walker 1954) and in conjunction with polygalacturonase and depolymerase probably break down cell wall constituents to yield pectic derivatives active in plugging the xylem (Waggoner and Dimond 1955). In addition to these enzymes, B-glycosidase has also been assigned a role in the wilt syndrome. Acting in conjunction with polyphenol oxidase it could account for vascular browning through hydrolysis and polymerization of phenolic constituents of the cell wall (Davis and Dimond 1954).

The role of mycelium and spores in physical obstruction of water flow has been questioned. Generally more vessels have been found free of mycelium than colonized in diseased plants (Brandes 1919; Rudolph 1922). Therefore it has been assumed they play only a minor role in wilt induction.

Toxins have also been implicated in vascular diseases of woody plants. When small elm trees were inoculated with fungus free culture filtrates of Ceratostomella ulmi Buisman, typical symptoms of Dutch elm disease were induced (Zentmyer 1942). Culture filtrates of this organism were fractionated into two toxic components, an alcohol insoluble polysaccharide and an unidentified ether soluble substance (Dimond et al. 1949). Both fractions induced different aspects of the disease. Using the same procedure similar substances from culture filtrates of E. fagacearum were isolated (White 1955). Other studies on this organism in vitro (Hoffman 1954;

Young 1949) resulted in demonstration of toxic substances. However, no evidence has been presented supporting existence of these compounds *in vivo*.

GENERAL METHODS

All studies involving physiology of oak,[1] tylosis induction, and host parasite relationships were carried out at the Iowa State Conservation Commission Nursery, Ames, Iowa or at Pilot Knob State Park, Forest City, Iowa.

Induction and Measurement of Tyloses in Red Oak

At the present time there is no evidence that tylosis formation arises in all cases from a universal, fundamental cause. Inducement through wounding, senescence, drought or disease suggests a common cause only inasmuch as it involves a disturbance of normal metabolic processes through cessation of normal sap flow. However, any information which could be obtained on aspects of tylosis formation, from whatever the cause, would ultimately prove helpful. In addition it was desirable to develop some method involving tylosis induction whereby metabolic products of both host and pathogen could be assayed for their ability to induce this specific response in the host. Therefore several experiments were performed to determine the response of red oak to wounding and to various chemical treatments.

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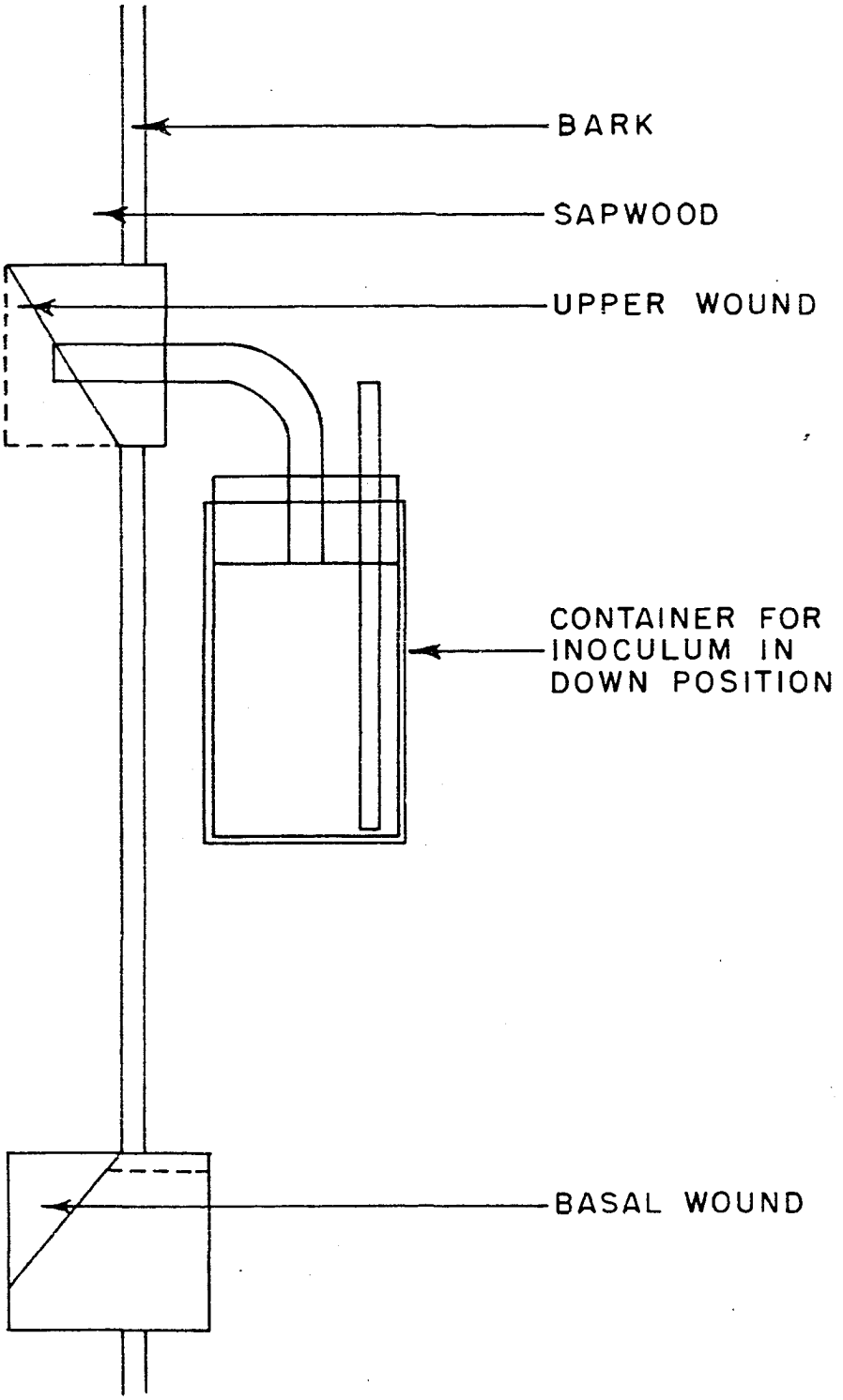
[1]The trees used in these studies were predominantly northern pin oak (Quereus ellipsoidales Hill). However no distinction was made between this species and northern red oak (Quereus borealis Michx.) or between their hybrids. The group is designated red oak throughout this study.

A standardized method was used in order that wound response could be placed on a semi-quantitative basis. Mature, healthy red oaks free from observable defects, were wounded by drilling two holes, separated by a vertical distance of 10 cm, into the bole of the tree by means of a brace and one-inch bit. Depending on the diameter, three to 10 pairs were drilled in each tree at waist height.

To observe the effects of wounding, sample cores were withdrawn with a Swedish increment borer from the midpoint of a vertical line joining the two holes, 36 to 48 hours after wounding. The cores were then stored in 90 per cent ethanol until examined for tyloses.

Various chemicals were tested for their effect on tyloses by injecting them into the vessels (Figure 1). By this means it was possible to fill the vessel lumina with a solution, seal it in, and after a given time interval withdraw a core with the increment borer for observation. Prior tests with a one per cent aqueous solution of crystal violet showed that water movement occurred initially through vessels of the current year's ring. However, due to the method that was used it was not possible to place the amount of solution taken up on a per vessel basis. Where movement of the test solutions through vessels did not take place immediately, due to air pockets, a small atomizer bulb attached to the air inlet on the container (Figure 1), was used to initiate

Figure 1. Device used for the injection of materials into red oak


BARK
SAPWOOD
UPPER WOUND
CONTAINER FOR
INOCULUM IN
DOWN POSITION
BASAL WOUND

flow.

To measure the extent to which vessels were occluded by tyloses, thin vertical sections from the current year's growth ring were cut from the core and observed under a calibrated binocular microscope. Vessel diameter was held constant for any one tree and the degree to which it was occluded by tyloses was expressed by a ratio R, such that

$$R = \frac{\text{horizontal extent of tyloses}}{\text{diameter of the vessel}} .$$

For any one sample, three vessel segments of equal diameter were selected and 20 tyloses in each vessel were measured. Tree to tree response varied considerably; therefore inter-tree responses were not comparable.

Analyses of Sap and Sapwood

Diseased and healthy sap and sapwood were compared as an initial step toward interpretation of the mechanism of wilting in diseased oak.

To study constituents of red oak sap initial attempts were made to obtain sap in the spring of the year by tapping. This method was not very successful although extended over a period of several weeks prior to, during and after bud break. A better procedure involved cutting young red oak twigs 20 cm long at the time the new growth ring was in the process of formation. Ends of cut sections were rinsed thoroughly in a stream of double distilled water. A hand atomizer was

connected by means of a rubber tube to one end of the twig and the sap expressed under pressure. Generally one or two drops were obtained from each twig. A 20 ml sample of the exudate was collected and evaporated down to 5.0 ml. *in vacuo* at 36°C. The concentrated sap was passed through a Dowex 50 x 8 cation exchange column in the hydrogen "cycle". After washing with 50 ml of distilled water, the cation fraction was eluted with 50 ml of four per cent NH_4OH, concentrated to 5.0 ml and chromatographed. The combined neutral and anion fractions were evaporated to 5.0 ml and passed through a Dowex 1 x 10 anion exchange column in the chloride "cycle". Elution was carried out with 50 ml of three per cent diethylamine, after washing with 50 ml distilled water. The neutral and anion fractions were concentrated to 5.0 ml and chromatographed.

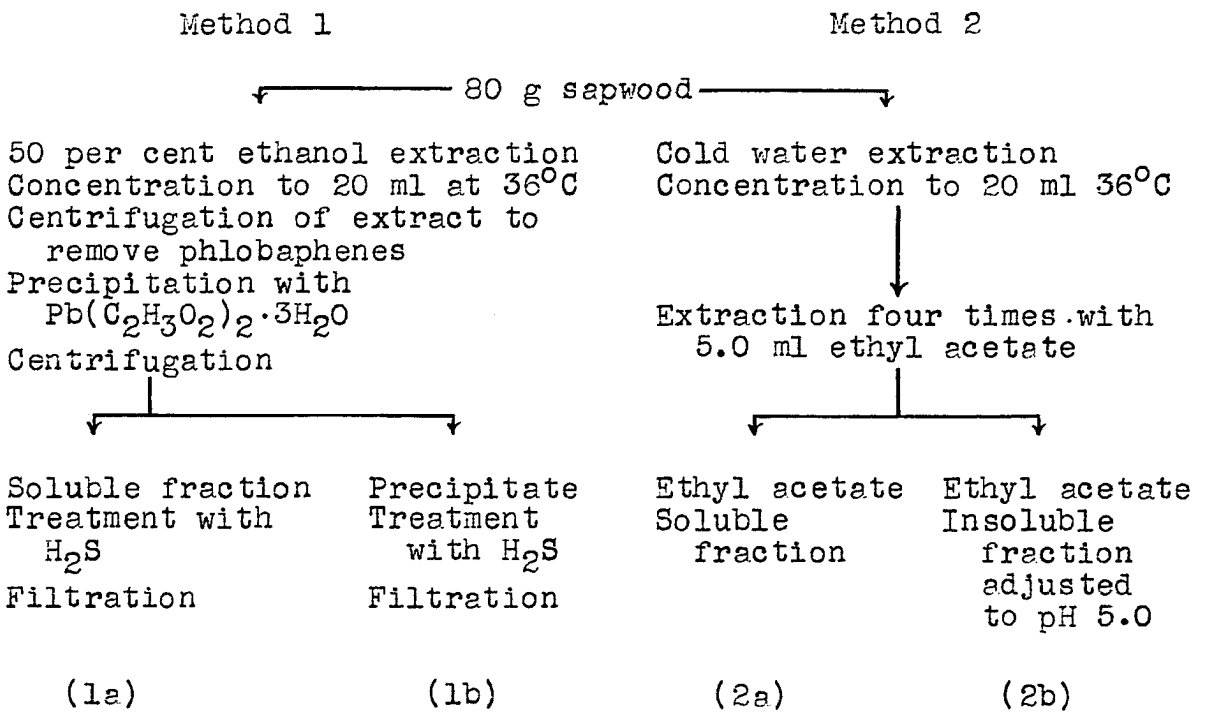
A different method was employed for diseased sap extraction. Several 20 cm sections from two-year-old twigs of red oak exhibiting advanced symptoms of the disease were washed and attached to a vacuum pump by means of a rubber hose. The free end was placed in a minimum of double distilled water and under a vacuum of 15 lbs/square inch approximately 1.0 ml was drawn through. The extracted solution was concentrated at 37°C *in vacuo* and chromatographed without further treatment. This simplified procedure was employed because nothing was known of the nature or stability of possible

toxic substances in diseased sap. During comparative studies with healthy red oak sap the latter was obtained and treated in an identical manner.

Sapwood samples from diseased and healthy trees were obtained with a brace and one-inch bit. Bark, phloem and heartwood were discarded when they became accidentally included in the sample. Diseased trees exhibited advanced leaf symptoms and generally vascular browning, although the latter phenomenon was not always observed. Samples were placed in air tight containers and stored at -12°C until extracted. Initial extraction procedures for isolation of sapwood constituents were 50 per cent alcohol at room temperature for 24 hours or cold distilled water at 5°C for 24 hours. Because preliminary extraction with either water or water-alcohol solutions resulted in isolation of a multitude of substances, further fractionation was necessary to purification and identification by chromatographic means. A procedure for the isolation of specific substances following extraction of sapwood (Figure 2) yielded four fractions from diseased and healthy sapwood which could be compared quantitatively and qualitatively by chromatography.

To study effects of various sapwood constituents on tylosis formation a different procedure was utilized in order to maintain the sapwood in a living condition as long as possible. Sapwood samples were taken from healthy trees

Figure 2. Procedure for the fractionation of aqueous and alcoholic extracts of sapwood


Method 1
Method 2
80 g sapwood
50 per cent ethanol extraction
Concentration to 20 ml at 36°C
Centrifugation of extract to remove phlobaphenes
Precipitation with $Pb(C_2H_3O_2)_2 \cdot 3H_2O$
Centrifugation
Cold water extraction
Concentration to 20 ml 36°C
Extraction four times with 5.0 ml ethyl acetate
Soluble fraction
Treatment with H_2S
Filtration
Precipitate
Treatment with H_2S
Filtration
Ethyl acetate Soluble fraction
Ethyl acetate Insoluble fraction adjusted to pH 5.0
(1a)
(1b)
(2a)
(2b)

by the method outlined previously and immediately placed in a container at 100 per cent relative humidity and 10°C for two to four hours until the extraction procedure given below was initiated. Sapwood samples taken in this manner are designated herein as fresh sapwood.

Prior tests had indicated that the initial water soluble constituents of healthy sapwood did not stimulate and apparently slightly inhibited the formation of tyloses. Also, it was shown that if there was a substance responsible for tylosis formation it was not formed immediately on wounding. Consequently, it was desirable to isolate a consecutive series of substances from the sapwood under conditions approaching those found in the wounded tree. To do this a continuous extractor was utilized (Figure 3). By this method 20 g samples of fresh sapwood chips were weighed rapidly and washed with distilled water. After draining, the samples were placed in each of two glass columns, 3.5 cm by 29 cm, and 10 ml of distilled water was added. By means of compressed air, a continuous flow of water over the samples was maintained at about 3.0 ml/minute. The bottom of the extractor was immersed in an ice bath. The extractives were withdrawn every two hours and replaced with equal quantities of distilled water. By this means consecutive samples could be withdrawn over an extended period of time. Samples taken were held at 0°C until injected into red oak

Figure 3. Continuous extractor used to fractionate sapwood constituents

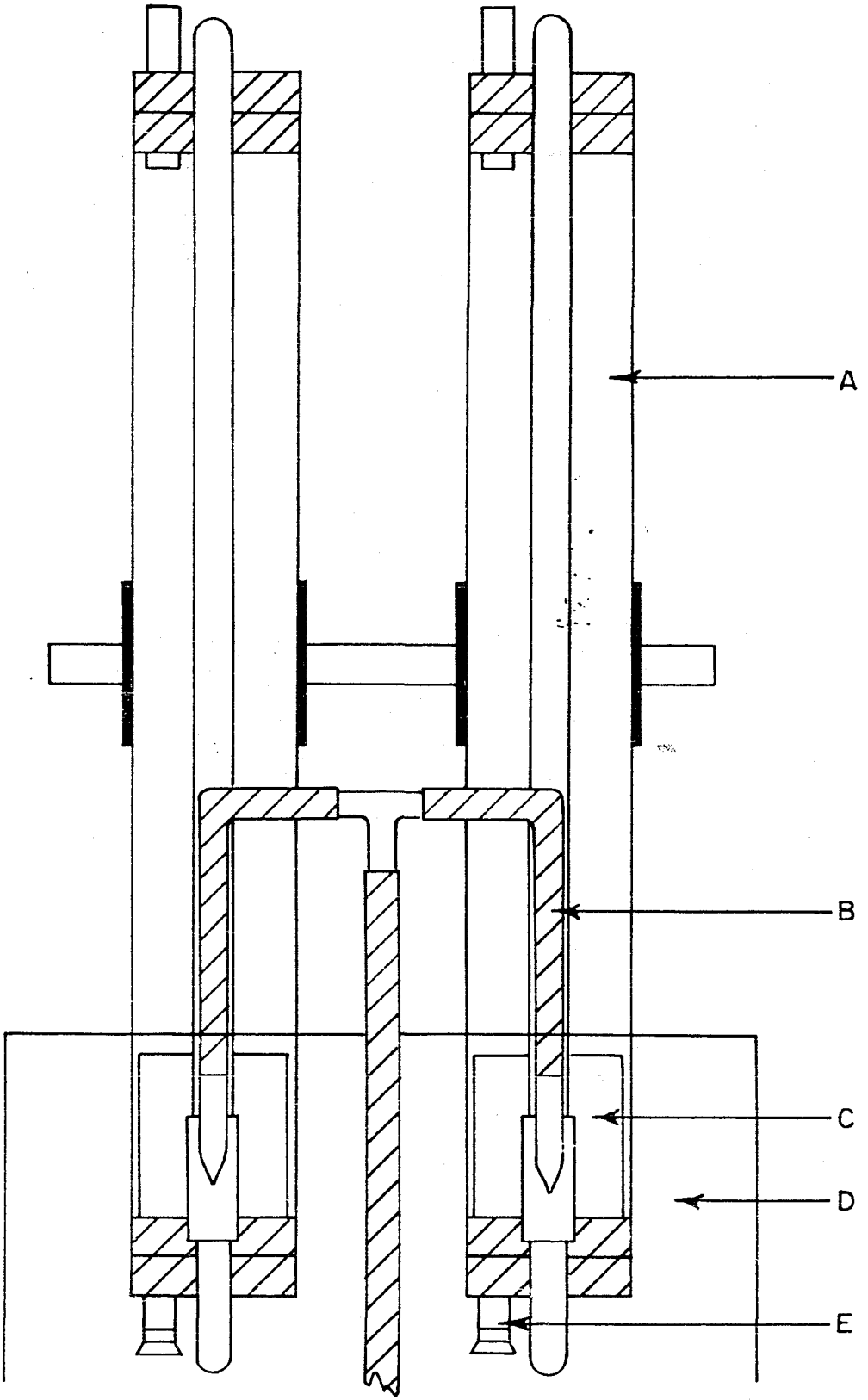
A. Column in which sapwood samples were placed

B. Compressed air inlet

C. Extractive reservoir

D. Ice bath

E. Drain for removing extractive


A
B
C
D
E

by the method indicated previously (Figure 1). The duplicate sample was reserved for chromatographic analysis.

Leaf Symptoms and Chemical Analyses

For observation and analysis of diseased leaves, 23 young 20- to 25-year-old red oaks were selected which had been inoculated artificially with E. fagacearum the previous year. Branches from each tree were tagged and the leaves were lettered alphabetically from the base. Observations of each leaf were recorded over a period of 60 days. Leaf symptoms were classified into four major groups:

Necrotic leaves - This symptom is identical in appearance to that described as summer or bronze-leaf symptoms (Engelhard 1955). Leaves so affected exhibited marginal necrosis which was delimited clearly from adjacent healthy tissue. Further necrotic development in leaves of young red oak was extremely slow. Characteristically, leaves exhibiting this symptom showed no further change until late in the growing season when within a period of a few days the leaves rapidly and uniformly became browned.

Bronze leaves - This was a characteristic leaf symptom on both young and mature diseased oaks. The leaves rapidly became dry, brittle and often faded to a bronze-green color. This appeared to be the result of an abrupt termination of the xylem sap flow. This possibility was supported further

by the fact that it did not appear in conjunction with other symptoms but typically was found affecting all leaves on a given branch.

Dwarf leaves - This leaf symptom generally observed in the spring has been described previously (Engelhard 1955; Engelhard and Bragonier 1957). The leaves were small and chlorotic but otherwise normal appearing. While a spring leaf symptom in mature diseased oak, it was observed in young oak as both a spring leaf symptom and later, after abscission of diseased leaves, as a result of premature secondary outgrowths of the axillary buds.

Chlorotic leaves - This symptom was characterized by extreme mottling or yellowing of the leaf. In general all leaves on diseased trees were paler than leaves from adjacent healthy trees.

For analyses of leaf tissue, standard analytical and chromatographic techniques were used. Total nitrogen was determined by the micro-Kjeldahl method, starch by the method of Widdowson (1932), and phosphorous by a modification of the Fiske-Subbarow method (Ward and Johnston 1953). Diseased leaves for analytical and chromatographic analyses included normal-appearing and chlorotic leaves. Necrotic, bronze, or dwarf leaves were not used.

Chromatographic Procedures

In routine analysis by chromatography a general procedure was adopted for study of various extractives. Chromatographic separation of mixtures was carried out at room temperature either by the ascending method on Whatman No. 1 or No. 3 paper, 8 1/2" by 8 1/2", or by the descending method on similar paper, 5 1/2" by 14 1/2". After the appropriate period of development, chromatograms were dried and exposed to UV light, 320 mμ, without treatment and after exposure to NH_3 fumes, to observe color changes in fluorescent substances. General chromogenic reagents and solvents are listed in Table 1.

Scopoletin for chromatographic comparison was isolated from oat seedlings, variety Victory (Goodwin and Kavanaugh 1949). It was purified chromatographically in BAW followed by elution and further chromatography with distilled water. Its UV absoprtion spectrum maxima in the Beckman DU spectrophotometer were identical to those reported in the literature (Goodwin and Kavanaugh 1949).

To prepare 3,4,dimethoxy cinammic acid, 10 mg of a methanolic solution of caffeic acid was made alkaline with a saturated solution of NaOH in methanol. To this solution, 0.5 ml of methyl iodide was added and the reaction mixture heated under reflux on a steam bath for 10 minutes. After cooling, the solution was concentrated, filtered and banded

Table 1. Common reagents and solvents used in chromatrographic procedures

ferric chloride ($FeCl_3$)	(Block et al. p. 228, 1955)
ninhydrin	(Consden 1948)
ammoniacal silver nitrate (ASN)	(Block et al. p. 132, 1955)
aniline hydrogen pthalate (AHP)	(Block et al. p. 133, 1955)
diazotized p-nitroaniline (DPNA)	(Block et al. p. 228, 1955)
phenol, 80 per cent	(Aronoff 1956)
n-butanol, acetic acid, distilled water (BAW) 50:3:10 v/v	
butyric acid, n-butanol, distilled water (BABW) 2:2:1 v/v	(Aronoff 1956)

on a descending chromatogram developed with 80 per cent phenol. The 3,4,dimethoxy cinnamic acid (Rf in 80 per cent phenol 0.85) was detected under UV, eluted in 0.5 ml of water and stored at 0°C for chromatographic comparisons.

In Vitro Studies on Endoconidiophora fagacearum Bretz

Culture medium

Although some strains of E. fagacearum appear to be partially heterotrophic with respect to biotin, thiamine and inositol (Beckman et al. 1953a), a chemically defined syn-

thetic medium, without added vitamins, was utilized to simplify subsequent analyses. The composition of the basal medium was based in part on studies of the nutritional requirements of E. fagacearum (Beckman et al. 1953a). The medium consisted of the following: Sucrose 34.2 g; L-arginine 3.48 g; KH_2PO_4 1.0 g; K_2HPO_4 1.3 g; $MgSO_4 \cdot 7H_2O$ 0.5 g; $FeSO_4 \cdot 7H_2O$ 10 mg; $ZnSO_4 \cdot 7H_2O$ 2.0 mg; $CaCl_2 \cdot 2H_2O$ 0.6 mg; $CuSO_4 \cdot 5H_2O$ 0.4 mg; $MnCl_2 \cdot 4H_2O$ 0.4 mg; $CoCl_2 \cdot 6H_2O$ 0.4 mg; $Na_2MoO_4 \cdot 2H_2O$ 0.2 mg; H_3BO_3 0.2 mg; and double-distilled water to make 1000 ml. The trace elements were prepared as stock solutions made up to volume with double-distilled water and stored at 5°C. Aliquots of 50 ml were dispersed in 250 ml, wide-mouth Erlenmeyer flasks and sterilized at 15 lbs pressure for 15 minutes. The organism was incubated at 22°C in still culture. Changes in chemical composition of the basal medium and varying methods of incubation are described under the respective experiments.

Inoculation of cultures and measurement of growth

All flasks for studies on growth measurements and growth promoting substances were inoculated with a 0.1 ml aliquot of a twice washed spore suspension of E. fagacearum by means of a calibrated sterile pipette. Spore suspensions were made from two- to four-week-old slants of the organism grown on potato-dextrose agar. In the case where growth promoting

substances in the culture medium were analyzed by means of the Avena coleoptile test, it was necessary to inoculate flasks through a seven-day period with the same quantity of inoculum. Therefore, the amount of inoculum was standardized turbidimetrically in a Klett-Summerson colorimeter by measuring a 2.0 ml aliquot and estimating the necessary dilution factor.

For estimation of growth in culture, media were filtered through a Buchner funnel on previously weighed filter paper. Mycelial mats were washed with 30 ml distilled water, dried at 95°C and weighed.

Measurement of growth promoting substances

For study of growth promoting substances produced by E. fagacearum in a chemically defined medium a previously described method (Nitsch and Nitsch 1956), with slight modifications, was employed. A clean, high-germinating sample of the oat variety, Nemaha, was used throughout the experiment. Seeds were first soaked in cold tap water for two hours. After soaking they were arranged uniformly in parallel rows, embryos up, between two sheets of soaked paper toweling. The sheets were then wrapped around large Pyrex test tubes, placed upright in a metal container and sealed with aluminum foil. The container was held in darkness in an incubator at 22°C for four days, at which time the coleoptiles were

approximately 20 to 30 mm. long. Under minimum white light, coleoptiles 20 to 25 mm long were selected and cut into 40 mm lengths, 30 mm from the tip. Ten coleoptile sections were placed in each sealed weighing bottle which contained 0.4 ml of a solution made by dissolving 1.0 mg $MnSO_4 \cdot H_2O$ in one liter of distilled water. These bottles were returned to the incubator in a sealed container for three hours. All coleoptile sections from a given sample were exposed five minutes to light during this procedure to standardize the method. After soaking, the $MnSO_4$ solution was removed rapidly from the samples with a syringe and replaced with the test reagents. These reagents had been mixed previously to reduce the time of exposure of the coleoptile sections to light. Each sample consisted of two lots of ten coleoptile sections in 0.1 ml buffer solution, 0.2 ml double distilled water and 0.1 ml of culture filtrate. Duplicate samples in the uninoculated medium were used as controls. The buffer mixture contained K_2HPO_4 1.794 g/liter + citric acid monohydrate 1.019 g/liter, pH 5.0 in double-distilled water (Nitsch and Nitsch 1956). The samples were placed in a sealed container and returned to the incubator for 24 hours. The individual coleoptile sections were measured with the aid of a calibrated binocular microscope. This method was standardized against known concentrations of indole acetic acid. Coleoptile section length plotted against the

log concentration of indole acetic acid was linear over the range from 1.0 to 100 µg/liter. Above this the increase in length was non-linear.

The basal medium and standard conditions of growth were used in all studies on coleoptile elongation. Prior to use, inoculated medium was passed through a Seitz filter to remove all mycelium and spores. The clear, culture filtrate was used directly without further treatment.

RESULTS AND DISCUSSION

Aspects of Red Oak Physiology in Relation to Tylosis Formation

Evidence up to this time has substantiated the theory that tyloses are a primary cause of reduced sap flow in xylem of diseased red oak (Beckman et al. 1953b). The evidence however was not conclusive and further information was sought to determine if tyloses were, in fact, a result rather than a cause of reduced water movement in the xylem.

Movement of sap in xylem of diseased and healthy red oak

It is well known in ring porous wood, that translocation of sap is confined generally to the outermost or current year's ring. To observe this in red oak, four- and five-year old branches were cut and immersed immediately in a one per cent aqueous solution of crystal violet. They then were placed under conditions conducive to high transpiration. Within two to three hours the dye was detected in petioles of the uppermost leaves. At this point the branches were removed from the solution, bark and phloem stripped off, and tangential and transverse free-hand sections made in order to observe distribution of the dye. Movement of the solution was restricted entirely to the outermost ring. All larger vessels, but not the tracheids or fibers, seemed to function

in translocation. The discrete vessel groups of the late wood were stained an intense blue throughout their length and could be observed clearly when the bark was removed. Very little anastomoses of the vessel groups were observed.

Diseased branches presented an interesting comparison. Whereas movement of the dye in healthy branches was distributed uniformly in discrete bundles throughout the circumference of the stem, in diseased branches movement was restricted to one or a few randomly distributed vessel groups. These functional vessel groups were generally free of tyloses while the remainder were occluded severely. Lateral translocation, that is movement of the dye at a point of occlusion to other non-occluded vessels or vessel groups, never was observed.

Furthermore, when branches from diseased and healthy trees were pruned to give an excess of leaf area on the diseased branch, vertical movement of the dye through the xylem was only one-fourth as rapid in the diseased branch in spite of an estimated 90 per cent reduction in functional vessels. From this it became apparent that leaves on diseased trees probably maintain themselves under conditions simulating a severe water shortage. The lower rates of water uptake of ten diseased leaves as compared to ten healthy leaves, measured over a 12-hour period, help explain the basis for, but not necessarily the cause of the reduced rate of dye

Table 2. Water uptake of diseased and healthy leaves over a 12-hour period

Leaf type	Water uptake ml/sq.cm/hour
Normal (check)	1.3×10^{-3}
Normal (diseased)	4.2×10^{-4}
Chlorotic	3.8×10^{-4}

movement in the xylem of diseased oak (Table 2).

Effect of reduced transpiration and drought conditions on tylosis formation in red oak

Defoliation of northern pin oak and the resultant reduction of xylem sap movement does not in itself induce tyloses (Beckman et al. 1953b). To substantiate this and further determine if tylosis formation under these conditions was merely a matter of time, several large branches up to 10 years old were maintained in a defoliated condition for periods up to 60 days. In no case were tyloses observed in excess of those found in leaf-bearing branches. On the other hand, in several mature red oaks suffering from drought injury during the summer of 1956, extensive tylosis formation was observed in the vessels of the bole and branches. Because the external symptoms of drought injury bore a strong resemblance to those of oak wilt, several attempts to isolate

the causal organism from these trees were made without success. This diagnosis was further supported by the fact that these trees were not located in areas where oak wilt had been observed previously. At the time of death tylosis formation and other vascular symptoms were identical in all macroscopic aspects to those found in infected trees.

Distribution and spread of tyloses in wounded red oak

Wounding initiated localized tylosis formation in red oak. Tyloses were observed as small balloon-like projections into vessel cavities 36 to 48 hours after wounding. Within five to seven days they had made contact with the opposite wall of the vessel segment.

Spread of tyloses about the wound site in relation to xylem sap movement was determined. Vertical spread was observed up to 40 cm beyond the wound site. Horizontal spread was restricted to approximately 2.0 cm on either side of the wound.

Radial distribution of tyloses was also determined. By careful control of wound depth only the first row of vessels was severed. Sampling with the increment borer to a depth encompassing five or six growth rings of the sapwood revealed tyloses in decreasing numbers inward from the second ring.

Prevention of tyloses at the wound site

Since the evidence presented was suggestive of the existence of specific hormones controlling tylosis formation, several attempts were made to interfere with the response by treatment of the wound site with various chemicals. Immediately after wounding, the area was washed thoroughly with specific substances sprayed on by means of a plastic squeeze bottle. Where materials other than water were used treatment was followed by a water rinse to limit the toxic effects of the chemicals. The experiment was run in duplicate (Table 3).

Table 3. Tylosis formation in red oak as affected by several chemicals

Treatment	Relative abundance of tyloses	R value
Water	+++	0.28
Acetone	+	0.18
$(NH_4)_2SO_4$ (50%)		0.00
Ethanol (95%)	++	0.25
Control	+++	0.27

Acetone and 50 per cent $(NH_4)_2SO_4$ limited tylosis size and reduced their relative abundance. Since these two chemicals also caused browning and partial disintegration of the wood tissue they were assumed to be toxic and were not

utilized further. The inability of water to affect tylosis size could be interpreted to mean either that a wound hormone did not exist or its release did not coincide directly with the time of wounding.

Since the inducing principle could arise as a delayed response to wounding, an experiment was set up whereby water was washed continuously through the wound at a constant rate of about 4.0 ml/minute for 24 hours. Controls, without continuous washing, were set up in the same tree. The experiment was run in duplicate. Sampling after 48 hours revealed extensive tylosis formation in controls but none in treated wounds.

Seasonal response to wounding in red oak

While in general tylosis formation as a response to wounding was initiated within a 48-hour period, a progressive diminution in tylosis size was observed throughout the growing season. Towards the end of September, 1957, tylosis formation was delayed considerably, while in October of the same year it could not be induced at all. There is perhaps a relationship between the cessation of tylosis formation and the onset of dormancy in the cambium.

Attempts at Characterization of the Mechanism of Tylosis Induction

The foregoing sections implied, not unequivocally, that the rise of tyloses in wounded tissue was caused by an abrupt termination of the normal flow of xylem sap through the vessels. Such an implication would provide a common basis for the origin of tyloses through wounding, senescence, disease or drought conditions.

Under this hypothesis, the xylem sap could have two possible functions in relation to tylosis induction. Occlusion during the growing season could result in the loss of nutrients essential for normal metabolism. Conceivably this might lead, in the presence of a sufficient quantity of energy yielding materials, to the formation of tyloses. As an alternative, xylem sap could have a dilution or inactivation effect upon a growth regulatory substance either normally present in living cells of the xylem or synthesized under abnormal conditions such as wounding. Much of the evidence supported the latter reasoning. Radial, horizontal and vertical spread of tyloses at a wound site appeared restricted to the area where normal flow of xylem sap had been obstructed. Tyloses occurred not only from the cells of the xylem rays which had been severed but also from those distant to the wound site. Therefore, tylosis formation was a response to a stimulus which could move radially along the chain of

living cells from one ray to the other through the cambial zone.

Effect of girdling to the cambium on tylosis formation

Transmission of any wound response in a vertical direction required that the stimulus be transmitted by non-living fibers, tracheids and apotracheal parenchyma. The only alternative was transmission through xylem rays to living cells of the cambium and from there to other rays. To test this reasoning a mature red oak was girdled to the depth of the cambium at two points 40 cm apart. A wound was made above, below and between the rings. After 48 hours samples were taken and examined for tyloses. Tyloses were formed abundantly above and below all wounds. From the wound made between the rings, tyloses extended beyond the upper ring. This suggested that if a specific growth-regulating substance was involved it could be transmitted through non-living tissue. Since tyloses occurred between the two rings it would seem that the stimulus was a product of wounded cells and was not translocated from some other site through the phloem.

Comparison of diseased and healthy sapwood constituents

Studies have suggested that the removal of a water soluble substance from the wound site prevented the formation of

tyloses. Attempts were made to isolate a regulatory substance which would control tylosis formation.

Water soluble constituents of infected and healthy sapwood were concentrated to 20 ml *in vacuo* at 36°C. Water insoluble material was removed by centrifugation and 200 µl were banded on a chromatogram developed ascendingly in BAW. Rf values and response to specific reagents of four compounds selected on the basis of quantitative or qualitative differences were studied (Table 4).

Table 4. General properties of compounds isolated from red oak sapwood

Compound letter	Rf values BAW	Relative abundance Diseased	Normal	Reagents ASN	$FeCl_3$	DPNA	UV Untreated	UV NH_3
A	0.45	+++		NR[a]	NR	NR	B	B
B	0.71	++	+	Br	B	fY	NR	NR
C	0.77	++	+	BG	GGr	B	Bk	Bk
D	0.91	++	+	NR	NR	P	GrY	bGrY

[a]B=blue Br=brown Gr=green Y=yellow b=bright
Bk=black G=grey P=purple NR=no reaction f=faint

Compound A was assumed to be a substance which arose as a direct result of host-parasite interaction since this material was not observed in healthy sapwood aqueous extracts. Subsequent extraction with 50 per cent ethanol, however, showed that compound A was also present in the extract of

normal sapwood. Therefore, all the substances observed in diseased sapwood extracts were present also in sapwood from healthy trees.

Thirty chromatographically identifiable compounds were observed originally on chromatograms of the sapwood extract. These substances interfered with chromatographic purification by limiting the amount of extract applicable to a chromatogram. Consequently, prior to chromatography, fractionation of the extract was undertaken.

Treatment of the solution containing compound A according to Method 1 (Figure 2) yielded a solution (1a) Method 1. Ascending chromatography in BAW gave an Rf value of 0.49 for compound A. After drying, compound A was located under UV, outlined, cut out and eluted in 1.0 ml of distilled water. After concentration in vacuo it was rebanded on a chromatogram and developed in 80 per cent phenol (Rf 0.79). Because the purity of the compound was in doubt, it was eluted again, banded on a chromatogram and subsequently developed in distilled water (Rf 0.79). The Rf values in 80 per cent phenol, BAW and distilled water, and its characteristic fluorescence under UV were properties similar to those reported for aesculin, 7-hydroxy, 6-glucoxy coumarin (Swain 1953). Purified compound A and an authentic sample of aesculin were hydrolyzed in 1.0 N HCL under reflux on a steam bath for three hours and chromatographed in BAW. A new bluegreen fluorescent sub-

stance corresponding to aesculitin, the aglycone of aesculin, was observed (Rf 0.78). Treatment of the chromatogram with ASN indicated the presence of a reducing substance corresponding to glucose (Rf 0.1). From these data, compound A was assumed to be chromatographically identical to aesculin.

Compound B (Table 4) was obtained by extraction of the sapwood by Method 2 (Figure 2). Concentration of the solution (2b) Method 2, yielded abundant compound B. When compound B was developed chromatographically in conjunction with tannic acid, Rf values in BABW (0.65), BAW (0.69) and 80 per cent phenol (0.24), and reactions to both ASN and $FeCl_3$ were identical. Compound B was assumed to be a substance either identical or closely related to tannic acid.

Compound C (Table 4) was isolated from sapwood of diseased and healthy trees by Method 2 (Figure 2). Concentration of the ethyl acetate soluble fraction (2a) Method 2, and chromatography in BABW yielded compound C in addition to several fluorescent and $FeCl_3$-positive substances. Compound C was eluted and developed chromatographically in conjunction with the simple phenols (phenol, catechol, resorcinol, hydroquinone, pyrogallol and phloroglucinol). In BAW (Rf 0.71), distilled water (Rf 0.72), and 80 per cent phenol (Rf 0.45), compound C reacted identically to an authentic sample of pyrogallol.

Compound D (Table 4) was isolated by the same method as

compound C. After chromatography in BABW (Rf0.73), it was located by its UV fluorescence and eluted from the chromatogram. On re-chromatography in NH_4OH: distilled water (pH 8.0) and in five per cent acetic acid, three fluorescent substances were observed (Table 5).

Table 5. Fluorescent compounds isolated by chromatography from the ethyl acetate fraction of diseased sapwood extracts

Compound number	Rf values		UV	
	5.0% acetic acid	NH_4OH:H_2O(pH 8.0)	Untreated	NH_3
1	0.50	0.68	B[a]	B
2	0.68	0.85	GrY	bGrY
3	0.81	0.90	P	bP

[a]B= blue Gr=green P=purple Y=yellow b=bright

Compound 2 (Table 5) which represented chromatographically purified compound D (Table 4) was eluted in a minimum of distilled water.

Color changes under UV on treatment with ammonia fumes are characteristic of flavanoid compounds, certain C_6C_6-type precursors (Geissman 1955) and their lactones (Swain 1953). Compound 2 (Table 5) exhibited fluorescent characteristics and Rf values similar to scopoletin (7-hydroxy, 6-methoxy coumarin) with which compound 2 (Table 6) was compared in

several solvents. The Rf values were not identical.

Ferulic acid, its methoxylated derivative (3,4-dimethoxy-cinnamic acid) and o-coumaric acid also were tested with negative results. The UV fluorescence spectrum of compound 2, using a Beckman DU spectrophotometer, was similar to the catechins (λ max. 200 and 275, λ min. 250 and 300). These substances, however, appear black under short and long wave UV (Geissman 1955). Compound 2 (Table 5) also exhibited no color change to yellow in visible light when exposed to ammonia fumes, a characteristic of many flavanols. Therefore, compound 2 (Table 5) was assumed to be a flavanoid perhaps structurally reflated to the flavanones.

Effect of sapwood extracts on tylosis formation

Growth regulatory substances arising as a delayed response to wounding could cause tylosis formation. In an attempt to isolate some tylosis-inducing substance, fresh sapwood samples were extracted under aerobic conditions (Figure 3). Preliminary chromatographic analysis of the extracts revealed a series of compounds up to 20 hours of extraction. Those compounds giving a response to $FeCl_3$ (phenolic substances) appeared instantaneously during the cold water extraction. After five hours no $FeCl_3$-positive reactions were observed. Ninhydrin-positive substances, having low Rf values, appeared during the two- to four-hour

extraction period. Extracted air-dried wood appeared to vary quantitatively and qualitatively from fresh sapwood extracts.

The two- to 20-hour extracts from fresh sapwood were injected into wounds in red oak. After 48 hours samples were withdrawn with an increment borer and examined for tyloses (Table 6).

Since only the 10-hour extract showed any marked stimulation to tylosis formation, this extract from the alternate sample was concentrated to 2.0 ml *in vacuo* at room temperature. Two descending chromatograms were developed in BAW until the solvent front reached 14 inches. One chromatogram

Table 6. Tylosis formation as affected by fresh sapwood extracts

Tree no.	Extract (Time of extraction in hours)	R value	Average length tyloses (u)	Standard deviation
1	2	0.17	53	4.58
	4	0.18	56	5.23
	6	0.18	55	4.45
	8	0.18	56	6.71
	10	0.22	68	4.53
Control		0.19	58	4.61
2	12	0.19	52	4.52
	14	0.20	53	5.34
	16	0.20	55	4.56
	18	0.21	56	5.21
	20	0.20	54	4.37
Control		0.21	57	3.42

was reserved for rough characterization of compounds present; the other was cut into strips corresponding to the Rf values, 0.1 to 1.0. Each strip was eluted with 3 ml of distilled water and the eluate diluted to 5.0 ml. The eluates were then injected into red oak. No stimulation of tyloses was detected. Substances present in the 10-hour extract were detected chromatographically (Table 7).

Table 7. Substances detected chromatographically after 10 hours of sapwood extraction

Rf	Reagents			UV	
	ASN	$FeCl_3$	Ninhydrin	Untreated	NH_3
0.05	NR[a]	NR	NR	bB	bB
0.07	Br	NR	NR	NR	NR
0.15	Br	NR	NR	NR	NR
0.25	Br	NR	NR	BGr	bBGr
0.60	NR	NR	NR	Y	bY
0.80	NR	NR	NR	B	B

[a]B=blue Gr=green NR=no reaction
Br=brown Y=yellow b=bright

Substances in diseased wood extracts differing quantitatively from healthy wood extracts were isolated to determine their effects on tylosis formation.

Compounds A, B, C and D (Table 4) were isolated from 200 g of sapwood chips. The four compounds were eluted and diluted to 5.0 ml with distilled water. In addition the partially purified fractions from the extraction procedure

(Figure 2) were tested. After extraction the four fractions (1a, 1b, 2a and 2b) were evaporated *in vacuo* to dryness, taken up in 10 ml distilled water and filtered. Filtrates were injected into wounds in red oak. The results suggested that none of the specific compounds or fractions was involved directly in tylosis formation (Table 8).

Table 8. Tylosis formation as affected by specific compounds and partially purified fractions isolated from the sapwood of diseased red oak

Compounds and fractions	R value
A	0.27
B	0.26
C	0.26
D	0.24
1a	0.26
1b	0.27
2a	0.28
2b	0.27
Control	0.28

No further attempts were made to isolate specific tylosis-inducing substances.

Chromatographic Analyses of Sap from Diseased and Healthy Red Oak

The general procedure for the extraction and separation of sap constituents has been described under methods.

Normal constituents of healthy red oak sap

The neutral fraction of healthy red oak sap was concentrated to 1.0 ml and 25 µl spots were applied to descending chromatograms developed in BABW. After development for 70 hours the chromatograms were dried, sprayed with AHP and heated for 10 minutes at 100°C. Three spots became evident. These were identified chromatographically as sucrose, glucose and xylose by descending co-chromatography with authentic samples and reactions with AHP, ASN and the molybdate reagent (Burrows et al. 1952). A fourth substance was observed which gave a positive reaction with the molybdate reagent (Burrows et al. 1952). The Rf value of this substance after prolonged development in BABW coincided with raffinose. An eluate of this spot was hydrolyzed in 1.5 N H_2SO_4 for two hours at 100°C, concentrated and re-chromatographed. This revealed, however, only two spots corresponding to glucose and fructose. In view of the superiority of xylose as a carbon source for growth of E. fagacearum in vitro (Beckman et al. 1953a), it is possible that this sugar has some role in the degree of specificity exhibited by this pathogen for its host.

The anion fraction, after concentration, was chromatographed ascendingly in 80 per cent phenol. When a chromatogram was sprayed with the molybdate reagent (Burrows et al. 1952) two yellow spots, characteristic of inorganic phosphate, were observed at Rf values of 0.32 and 0.65.

To further characterize these spots a banded chromatogram was prepared (Whatman No. 1 paper, previously washed thoroughly in 0.1 M citric acid) and developed in 80 per cent phenol. After complete drying the chromatogram was cut into strips corresponding to Rf values from 0.1 to 1.0, eluted with 1.0 ml of double-distilled water and added to test tubes containing 9.0 ml of the basal medium less phosphorous. The potassium was supplied 0.018 M KNO_3. A control sample was prepared from a chromatogram on which none of the anion fraction had been banded. An aliquot of a heavy spore suspension of E. fagacearum, which had been washed three times in double-distilled water, was added to each test tube. These cultures were incubated on a wrist shaker at 28°C. After 48 hours, the fungus growth was estimated turbidimetrically in the Klett-Summerson colorimeter (Table 9). The results indicated that the two spots were probably ionic species of phosphate.

Aliquots of 25 µl of the cation fraction were applied to each of several chromatograms and separated two dimensionally by the ascending technique in 80 per cent phenol and BABW. After drying, one chromatogram was sprayed with ninhydrin and heated at 100°C for 10 minutes. Fourteen ninhydrin-positive spots were found. Their positions were marked on the remaining chromatograms, the spots were cut out and eluted in a minimum of distilled water. Eluates were

Table 9. Growth of E. fagacearum on addition of chromatographic eluates of the anion fraction to the basal medium[a]

Rf of chromatograms	Klett reading	
	Anion fraction	Control
0.1	38	35
0.2	41	42
0.3	89	41
0.4	65	38
0.5	46	42
0.6	83	42
0.7	79	39
0.8	41	34
0.9	39	31
1.0	39	36

[a]Phosphorus deleted

applied individually on descending chromatograms against standards suggested by a previously prepared chromatomap. In this manner glutamine, glutamic acid, asparagine, methionine, alanine and glycine were identified chromatographically. Tentatively identified were tyrosine, aspartic acid, and threonine. The presence of glutamine, asparagine and glutamic acid in relatively high concentrations was interesting since they also were observed to be among the predominant nitrogeneous compounds in the xylem sap of apple trees (Bollard 1953).

In order to determine if indole compounds, particularly tryptophan, were present another chromatogram was sprayed

with 0.5 per cent alcoholic $NaNO_2$ and exposed to HCl fumes in a closed container (Block et al. 1955). No reaction was observed.

Evidence has been presented in the literature that phosphoryl choline is an organic form of phosphorous found in the xylem sap of barley seedlings (Maizel et al. 1956). When concentrated red oak sap, without further treatment, was applied to a chromatogram and developed according to the method used in these barley studies the presence of phosphoryl choline could not be demonstrated.

Constituents of vessels of diseased red oak

The "sap" obtained by siphoning double-distilled water under low vacuum through stem sections of diseased red oak was chromatographed by the ascending technique on Whatman No. 1 paper. Aliquots of 25 µl of these vessel constituents from healthy and diseased trees taken under identical conditions were applied side by side for comparison. The chromatograms were developed in BAW (Table 10). Marked differences existed between the vessel constituents of diseased and healthy red oak. The presence of phenolic compounds, indicated by a positive reaction with $FeCl_3$, in the vessels of diseased oak parallels results with tomato wilt (Davis and Waggoner 1953).

Subsequent attempts to identify the compounds found in the xylem vessels revealed that compound G (Table 10) was

Table 10. General properties of water soluble vessel constituents

Compound letter	Rf value BAW	Normal	Diseased	Reagents				UV	
				ASN					
				25°C	100°C	$FeCl_3$	AHP	Untreated	NH_3
E	0.1 to 0.2	+	+	NR[a]	Br	NR	Br	NR	NR
F	0.17		+	G	GBl	GB	NR	NR	NR
G	0.50		+	NR	fBr	NR	NR	B	B
H	0.65		+	Br	Br	B	NR	NR	NR

[a]B=blue Br=brown NR=no reaction
Bl=black G=grey f=faint

identical to compound A (Table 4) identified as aesculin. Compound H (Table 10) was identified as tannic acid, compound B (Table 4).

Compound F (Table 10) was not observed in the sapwood extracts (Table 4) although no concerted attempt was made to analyze all the fractions.

A blue reaction with $FeCl_3$ is indicative of the 3, 4, 5, trihydroxy grouping in the B-ring of polyhydroxy flavanoids (Geissman 1955; Roberts et al. 1951). Since compound F (Table 10) was not similar in reaction to specific reagents or in Rf values to the simpler phenols, it was classified as a flavonoid compound.

Leaf Symptoms on Diseased Red Oak

Severely infected trees exhibited many symptoms indicative of nutrient deficiencies. Growth generally was retarded and dwarf leaf symptoms together with necrotic and chlorotic leaves were abundant. Distribution and rate of spread of leaf symptoms were observed in an attempt to ascertain if the symptoms could be attributed to toxins or to failure of essential metabolites or nutrients to reach the leaf.

Comparative rates of sap flow in healthy and diseased red oak

Three-year-old branches from healthy and diseased trees, free from defects and of nearly equal diameter, were selected

at the time when leaf development was progressing rapidly but the current year's growth ring was not observable. Water carrying capacities of the branches were measured using the method employed by Beckman et al. (1953b), with the exception that a one percent solution of crystal violet rather than water was used. The quantity of dye passing through a 10 cm section in 10 minutes was recorded (Table 11).

Table 11. Comparative water carrying capacities of diseased and healthy branches

Time in days from first observation	Water carrying capacity ml/minute	
	Diseased	Healthy
0	0.000	0.280
6	0.040	0.289
15	0.110	0.270
20	0.110	0.315
27	0.120	0.350

In both diseased and healthy branches the water carrying capacities increased with the appearance of new vessels. The flow rate in diseased sections, however, was severely restricted. An examination of cross-sections revealed the following: 1) The previous year's growth ring which functions initially in sap transport was largely occluded in diseased sections. 2) Occlusion of new vessels was evident within a few days after formation.

Function of the old growth ring in initial transport of

xylem sap probably bears an important relationship to symptom development in trees which survived infection from the previous year. From the time leaf development begins in the spring, a partial to severe restriction in sap flow limits the movement of essential inorganic and organic materials.

Distribution and rate of spread of leaf symptoms

Observations made on the distribution and spread of leaf symptoms led to the hypothesis that symptoms arose as a result of a deficiency of nutrients or other metabolic constituents (Table 12). The primary symptom on young infected red oaks was premature abscission of leaves. In conjunction with this axillary buds of abscissed leaves often developed prematurely and gave rise to typical dwarf leaves. Abscissed leaves frequently were devoid of specific symptoms prior to abscission. It should be emphasized however that in relation to leaves on adjacent healthy trees, leaves prior to abscission were always a lighter green and devoid of lustre.

Marked chlorosis was observed. In some cases necrotic development followed chlorosis but in general chlorosis was independent of other symptoms.

Necrotic symptoms while by no means numerous relative to other symptoms were important with respect to possible existence of specific toxins. While necrotic symptoms might have arisen as a result of the action of toxic substances,

Table 12. Distribution and rate of development of leaf symptoms on young diseased red oak

	Days from June 20, 1956											
	0	2	4	8	12	15	22	29	32	36	58	62
Branch 1 (Tree 1)[a]												
Total leaves	16	16	16	16	14	14	14	15	13	12	11	8
Normal leaves	15	15	15	15	13	13	13	12	10	7	4	1
Symptom distribution												
Necrosis	C[b]											
Chlorosis								H			NOP	JK
Abscission					FG				DI	AEB	H	COL
Dwarf leaf								F		GI		
Branch 2 (Tree 2)												
Total leaves	13	10	10	10	11	10	10	9	9	9	9	8
Normal leaves	10	7	7	6	5	4	4	1	1	0	0	0
Symptom distribution												
Necrosis	AFG							M			H	
Chlorosis				M	L			HK				
Abscission		BEJ				D		CF		I		K
Dwarf leaf					B			D		C		
Branch 3 (Tree 3)												
Total leaves	22	22	21	21	21	21	20	18	18	15	10	9
Normal leaves	18	18	17	17	17	17	17	15	12	8	5	4
Symptom distribution												
Necrosis	AGL									DJ		
Chlorosis	R							N	JK	O		
Abscission			T				R	AS		BFM	CEHJL	P
Dwarf leaf												

[a]Data selected as typical from observations on 23 trees.

[b]The alphabetical designation fixes the position of the leaves relative to the base of the stem.

their random distribution, slow rate of development and scarcity of numbers tended to refute this interpretation. In addition, the rate of spread of initial necrotic areas on individual leaves was extremely slow on those which remained on the tree. Further progression, determined by outlining the necrotic margin with India ink, took place only towards the end of the growing season. At this time a rapid browning of the whole leaf occurred. This was analagous to that observed on healthy trees later in the season.

In an attempt to clarify the cause of dwarf-leaf development on diseased red oak, several diseased branches with terminal dwarf leaves, in addition to typical necrotic or chlorotic leaves, were placed in solutions of 1.0 per cent aqueous crystal violet for 24 hours, at which time the bark was removed and the branches cut into sections. Restricted dye movement occurred to all necrotic and chlorotic leaves but not to the dwarf leaves. Furthermore, when 10 cm sections of the apical portion of stems containing dwarf-leaves were connected to a vacuum pump, dye could not be drawn through any of the sections at a vacuum of 30 lbs/square inch. This indicated that dwarf leaves subsisted only on the nutrients and water available in the upper portion of the stem, or which could be imbibed through non-occluded micro-capillaries of the xylem.

The bronze-leaf symptom generally was not observed in

close proximity with other symptoms, but overtook all the leaves on a particular branch, or section of a tree. Complete occlusion of the stem could not be demonstrated in all cases but the symptom was typical of an abrupt termination of water movement in the stem. A condition similar to the bronze-leaf symptom could be induced by severing the conducting vessels of a normal branch.

Distribution of E. fagacearum in leaves in relation to leaf symptoms

The presence of the organism in necrotic and chlorotic leaves and its absence in normal appearing leaves would involve the organism directly in the symptom complex. This conclusion had been reached previously when it was observed that the organism could not be isolated from other than symptomatic leaves on diseased trees (Young 1949). To test this hypothesis further, leaves exhibiting each of the four typical symptoms were collected from young diseased red oak. Petioles were removed, sterilized in 30 per cent Chlorox for one minute and plated on potato-dextrose agar. After incubation for two weeks at 22°C the petioles were examined for the presence of E. fagacearum (Table 13).

Percentage infection in normal-appearing leaves was contrary to earlier observations (Young 1949). When examined in association with other data (Table 12), the results were difficult to reconcile with any theory associating necrotic

Table 13. Presence of E. fagacearum in petioles from normal-appearing and symptomatic leaves from diseased trees

Symptoms	Number of petioles examined	Number infected	Percentage infected
Necrotic leaves	205	168	82
Chlorotic leaves	185	135	73
Bronze leaves	80	52	64
Normal-appearing leaves	192	144	75

development directly with E. fagacearum. Observed differences in percentage of infection between necrotic and normal-appearing leaves, while small, were probably real and indicative of the extent of vascular infection and therefore indirectly, of vascular occlusion.

Chromatographic and analytical comparisons of diseased and healthy red oak leaves

A general procedure was used in preparing leaf extracts for chromatography. Portions containing 25 g of diseased or healthy leaves, which had been rinsed previously in tap water, were ground for two minutes in a Waring blendor with 180 ml of 85 per cent ethanol. The leaf extract was filtered and extracted three times with 20 ml portions of petroleum ether (Skelly B). This fraction was discarded. The 85 per cent

ethanol soluble component was evaporated down at 35°C to 40 ml and filtered. This solution was designated as Fraction A. Further treatment varied according to the nature of the experiment.

Flavonoid and phenolic constituents. Fraction A was evaporated down to 10 ml in vacuo. Quantities of 5 to 25 µl of both healthy and diseased fractions were examined by two dimensional chromatography in 80 per cent phenol and BABW in order to establish quantitative or qualitative differences between healthy and diseased leaves (Table 14).

Previous evidence has indicated that flavonoid compounds with catechol groupings gave a green color with $FeCl_3$ and those with phloroglucinol groupings a blue color (Roberts and Wood 1951). However, the green color given by the ortho-dihydroxy flavonoids was not specifically diagnostic (Geissman 1955; Clarke and Nord 1955). While no attempt was made at the time to identify specific compounds, all simple phenols giving a blue color with $FeCl_3$ had relatively high Rf values in BABW and 80 per cent phenol. Consequently, it was assumed that most of the compounds listed (Table 14) were the more complex flavonoid compounds. From the preliminary data (Table 14), there appeared to be a qualitative excess of phloroglucinol-derived flavonoids in the diseased leaves and a deficiency of catechol-derived flavonoids.

Presence of an excess of phloroglucinol-derived flavo-

Table 14. Phenolic and flavonoid constituents of diseased and healthy red oaks

Compound number	Rf values BABW	Rf values 80% phenol	Relative concentrations Diseased	Relative concentrations Healthy	Reagent $FeCl_3$	UV Untreated	UV NH_3
1	0.00	0.00	+	+	B[a]	NR	NR
2	0.12	0.12	+	+	B	NR	NR
3	0.12	0.21	+		B	NR	NR
4	0.14	0.21	+		B	NR	NR
5	0.18	0.31		+	B	Bl	OY
6	0.25	0.43	+		B	NR	NR
7	0.28	0.18	+	+	B	NR	NR
8	0.30	0.31	+		B	NR	OY
9	0.31	0.18	+		B	NR	NR
10	0.40	0.62	+	++	Gr	NR	OY
11	0.56	0.43		+	Gr	Bl	OY
12	0.56	0.50	+	++	Gr	Bl	Gr
13	0.56	0.81	+	+	NR	B	bB
14	0.68	0.90		+	NR	NR	bP
15	0.70	0.63		+	fGr	B	bB

[a]B=blue Gr=green P=purple b=bright
Bl=black OY=orange yellow NR=no reaction f=faint

noids in diseased red oak leaves suggested that some of these compounds might have been translocated from infected vessels of the xylem. Consequently 25 µl spots of diseased oak sap and Fraction A of diseased leaves were applied at varying concentrations to descending chromatograms. After development in BABW the chromatograms were dried, observed under UV and afterwards sprayed with $FeCl_3$. Of the compounds found in the vessels of diseased sapwood none was chromatographically identical to any of the compounds found in diseased leaves (Table 15).

From this information two conclusions were drawn. Either substances observed in the vessels of diseased oak represented contents of non-functional conducting elements or the phenolic constituents of the xylem were being enzymatically altered in leaves without prior observable accumulation.

To obtain information on this latter possibility, the three compounds (F, G and H) from the xylem "sap" (Table 10) were separated and purified by successive chromatography in 80 per cent phenol and BAW. The substances were eluted after thorough drying of the chromatogram and made up to 5.0 ml. Eight healthy red oak leaves were allowed to take up the solutions while control leaves were placed in distilled water. After three days there was no macroscopic evidence of toxicity. Individual leaves were ground in 85 per cent ethanol, with a mortar and pestle, filtered, concentrated and chroma-

Table 15. Comparison of phenolic substances in leaves and xylem of diseased red oak

Compound number	Observed		Rf values	Reagents			UV	
	Leaves	Xylem	BABW	ASN 25°C	ASN 100°C	$FeCl_3$	Untreated	NH_3
1	+		0.00	Br[a]	BrBl	B	NR	NR
2		+	0.12	G	GBr	GB	NR	NR
3	+		0.16	Br	Br	B	NR	NR
4	+		0.25	Br	Bl	B	NR	NR
5	+		0.31	BrBl	B	B	NR	NR
6	+		0.43	Br	B	Gr	NR	O
7	+		0.46	Br	Br	B	NR	NR
8		+	0.48	NR	NR	fGr	B	B
9		+	0.61	Br	Br	B	NR	NR

[a]B=blue Br=brown Gr=green NR=no reaction
Bl=black G=grey O=orange f=faint

tographed. The three compounds were re-isolated from the leaves, unaltered. Other than the three substances mentioned no other qualitative or quantitative differences were observed between control leaves and those placed in the chromatographic eluates. This information supports the conclusion that the substances designated herein as xylem "sap" constituents of diseased red oak were soluble compounds from nonfunctional or occluded vessels and hence were not translocated to the leaves.

To separate flavonoid constituents from aromatic acids, fraction A was acidified to pH 2.0 in an ice bath with 5.0 N chilled HCl. The acidified solution was saturated with NaCl and extracted six times with 5.0 ml portions of ethyl acetate. The ethyl acetate fraction was separated by centrifugation and extracted thoroughly five times with 10.0 ml of 5 per cent $NaHCO_3$. The salt solution was decanted, re-extracted with 5 ml portions of ethyl acetate, cooled and acidified to pH 2.0 in an ice bath with 5.0 N HCl. This fraction was re-extracted three times with 5.0 ml of ethyl acetate. The ethyl acetate solution was concentrated to dryness *in vacuo*, dissolved in 1.0 ml distilled water and chromatographed two dimensionally in 80 per cent phenol and BABW. From this fraction, which varied somewhat in composition, three prominent fluorescent compounds were consistently observed (Table 16).

Earlier chromatographic data (Swain 1953) suggested that compounds one and two (Table 16) had properties similar to chlorogenic and caffeic acid, respectively. One mg of an authentic sample of chlorogenic acid was hydrolyzed under reflux on a steam bath with 1.0 N HCl for one hour. The HCl was removed from the solution by evaporation to dryness several times. After dissolving in a minimum of water, the hydrolysate was chromatographed in BABW and the caffeic and chlorogenic acid spots located under UV. The spots were

Table 16. Phenolic acids from diseased and healthy leaves of red oak

Compound number	Rf values BABW	Rf values 80% phenol	Relative concentrations Diseased leaves	Relative concentrations Healthy leaves	Reagents ASN	Reagents $FeCl_3$	UV Untreated	UV NH_3
1	0.40	0.50	+	++	Br[a]	fGr	fB	BGr
2	0.56	0.50	+	++	Br	NR	B	bB
3	0.68	0.75		+	Br	Gr	B	B

[a]B=blue Gr=green b=bright
Br=brown NR=no reaction f=faint

eluted in 0.5 ml distilled water and chromatographed separately in 80 per cent phenol and BABW, with the leaf extracts. Rf values and reaction under UV of compounds one and two (Table 16) were identical to chlorogenic and caffeic acids, respectively.

Quinic acid, the third product of acid hydrolysis of chlorogenic acid was not found in the leaf samples. It was located in the hydrolysate of the authentic sample by spraying with the molybdate reagent (Burrows et al. 1952). It formed a white spot on a blue background. The observation of quinic acid in diseased leaves would have suggested that enzymatic hydrolysis of chlorogenic acid had occurred and that the acid was not localized in the cell vacuole. This would have explained the smaller concentration of chlorogenic

acid observed in diseased leaves. Since no quinic acid was detected in diseased or healthy leaves, however, the lower quantity of chlorogenic acid observed in diseased leaves was assumed to be a result of metabolic deficiencies.

Normal metabolic constituents. For both normal and diseased leaves Fraction A was evaporated to 10 ml *in vacuo* at 32°C and passed through ion exchange columns. The cation fraction was treated as indicated in the section on methods. The anion fraction was isolated on an IRA 400 column in the hydroxyl form and eluted with 0.1 N HCl. After separation, the cation, anion and neutral fractions were concentrated to 5.0 ml *in vacuo* at 32°C and chromatographed.

Twenty-five µl samples of the cation fraction from both diseased and healthy leaves were chromatographed two dimensionally in 80 per cent phenol and BABW. After drying, chromatograms were sprayed with ninhydrin and heated at 100°C for two minutes. The healthy leaf fraction contained 16 ninhydrin-positive spots. Using a previously prepared chromatomap and known amino acids, positive chromatographic identification was made of the following amino acids: glycine, asparagine, glutamine, proline, alanine, lysine, arginine and valine. Glutamic acid and serine were tentatively identified. At similar concentrations no ninhydrin-positive substances were isolated from the diseased leaf fraction.

Twenty-five µl samples from the anion fraction of

diseased and healthy leaves were developed chromatographically in 80 per cent phenol and BABW. After drying, the chromatograms were sprayed with one per cent dextrose and then with ASN (Gore 1951). Eight non-fluorescent spots were recorded from healthy leaf extracts. Two were identified as citric acid and malic acid by co-chromatography with authentic samples. Diseased leaf extracts contained no definitive spots at this concentration.

Neutral fractions of diseased and healthy leaf extracts were applied in 25 µl aliquots to descending chromatograms. The chromatograms were developed for 70 hours in BABW, dried, sprayed with AHP and heated at 115°C for five minutes. Three AHP-positive spots were recorded for extracts from healthy leaves. By descending co-chromatography of these extracts with sucrose, fructose and glucose in BABW and BAW, all three sugars were identified. None was observed in diseased leaf extracts at this concentration.

These chromatographic analyses have indicated deficiencies of essential metabolites in diseased leaves. Identified compounds in healthy leaves were common glycolytic, Kreb cycle, or amino acid constituents. Their complete absence in diseased leaves at concentrations where they were normally observed in healthy leaves was conceivably more indicative of acute starvation than of toxic activity of the pathogen or of host-parasite interaction.

Starch accumulation in diseased leaves. Necrotic, chlorotic and normal appearing leaves from diseased red oak were cleared in boiling 80 per cent ethanol and immersed in an I_2KI solution. These leaves had accumulated starch in excess of that found in healthy leaves. Excised leaves from both healthy and diseased trees were placed in a moist chamber for 48 hours in darkness and then tested for starch. Healthy leaves gave a negative reaction while diseased leaves retained a strong positive reaction. The assumption was made from these observations that the mechanism for starch degradation had been obstructed. Since chromatographic evidence indicated a deficiency of glucose in diseased leaves, the inhibition of starch breakdown could not be attributed to an accumulation of this sugar.

The reversible enzymatic breakdown of starch to glucose-one-phosphate by phosphorylase has been reported to proceed towards the formation of glucose-one-phosphate as the pH increases (Hanes 1940). The possibility that a pH optimum contols the breakdown of starch led to a study of the phosphorylase activity of oak leaves. Solutions made by grinding 25 g samples of diseased and healthy leaves in 100 ml of distilled water were used to estimate the pH values. Healthy leaves had a pH of 5.6, diseased leaves a pH of 4.5. Determination of phosphorylase activity in healthy leaves followed the method of Sumner et al. (1950). One to 5.0 ml of crude

leaf extracts containing cytoplasm and chloroplasts were prepared by grinding 2.5 g of leaves at $0^{o}C$ in 10.0 ml of a mixture of washed white sand and chilled 0.1 M citrate buffer pH 6.0. The crude extract was filtered through several layers of cheese cloth and centrifuged to remove sand and cell debris. To the filtered extract were added 0.1 ml of a glucose-one-phosphate buffer substrate, 0.5 ml starch solution, 0.5 ml of 10^{-3}M $HgCl_2$ to inhibit amylase activity and 0.5 ml toluene. Analyses of healthy leaf extracts for inroganic phosphate by a modified Fisk-Subbarow method (Ward and Johnston 1953) gave a maximum yield after 10 hours of 12.0 µg of inorganic phosphorus. The diseased leaf extracts were inactive. Due, however, to the low activity of the enzyme in the crude extracts of healthy leaves this aspect was not investigated further.

Chemical analyses of leaves. Since starch accumulation was a common symptom of nitrogen or phosphorus deficiency, diseased and healthy leaves (see Methods) from adjacent trees were analyzed at three periods through the summer of 1957. Leaf samples were pooled, washed thoroughly in distilled water, dried, ground in a Wiley mill and analyzed (see Methods). Chromatographic and chemical analyses indicated chlorotic and necrotic symptoms arose from a deficiency of essential mineral and organic constituents (Table 17).

Table 17. Chemical analyses of diseased and healthy leaves of red oak

Leaves	Dry weight in g	Alcohol insoluble residue in g	Reducing sugar in insoluble residue in g	Percentage[a] nitrogen	Percentage ash	Percentage phosphorus
Healthy	5.0000	2.8389	0.748	2.15	3.76	0.0019
Diseased	5.0000	2.7061	0,892	1.64	3.35	0.0010

[a]All percentages are expressed on the basis of the dry weight and are averages of four determinations.

Metabolism and Metabolic Products of *E. fagacearum*

Effect of certain phenols on the growth in vitro of *E. fagacearum*

Presence of phenolic substances in aqueous extracts of diseased wood (Table 4) and in vessels of diseased red oak (Table 10) prompted an investigation on the effect of phenols on growth *in vitro* of *E. fagacearum*. Phenolic compounds were added at three concentrations in an attempt to find both an inhibitory and a stimulatory range. The medium used differed slightly from the basal medium. It consisted of 2.64 g/liter l-asparagine and 0.05 M sucrose, except in the case of the medium containing tannic acid where 0.1 M sucrose was used. Culture flasks were randomized and held at room temperature for the duration of the experiment. Growth was recorded as the average mycelial dry weight from two flasks.

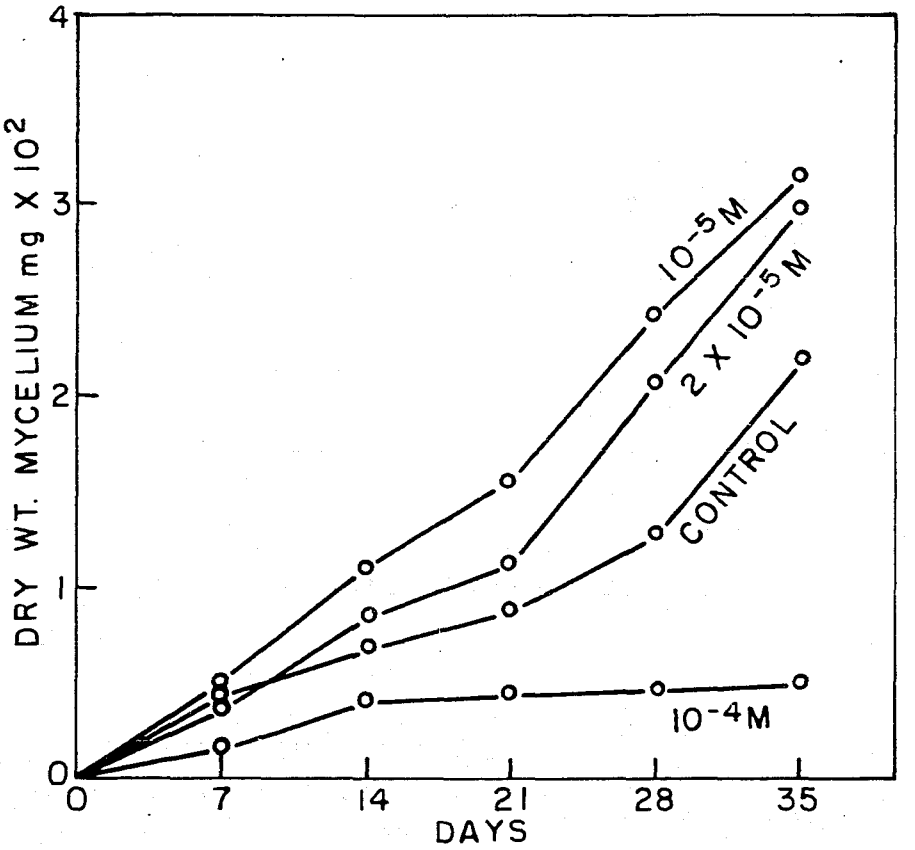
All phenolic compounds at concentrations of 10^{-5} M with the exception of phloroglucinol, exhibited stimulatory effects on the *in vitro* growth of *E. fagacearum* (Figures 4, 5, 6, and 7). This suggested a possible stimulatory role for phenolic compounds in the wilt syndrome.

Effect of culture filtrates on tylosis formation in red oak

Specific substances synthesized by the organism may have a role in tylosis induction (Beckman *et al.* 1953a). To

Figure 4. Growth of E. fagacearum as affected by tannic acid

Figure 5. Growth of E. fagacearum as affected by phloroglucinol


DRY WT. MYCELIUM mg X 10²
4
3
2
1
0
0
7
14
21
28
35
DAYS
10⁻⁵M
2 X 10⁻⁵M
CONTROL
10⁻⁴M

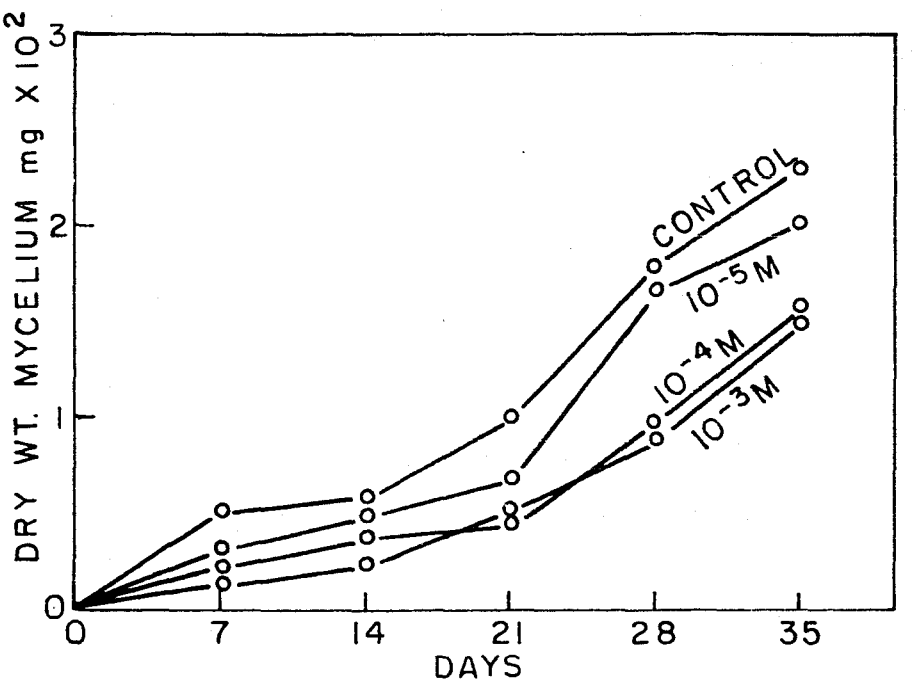
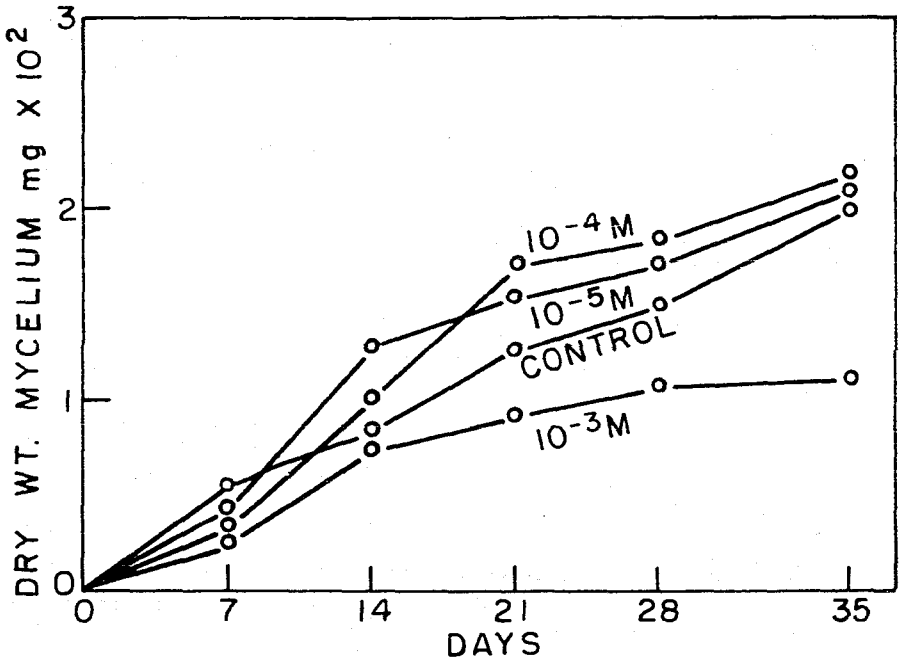
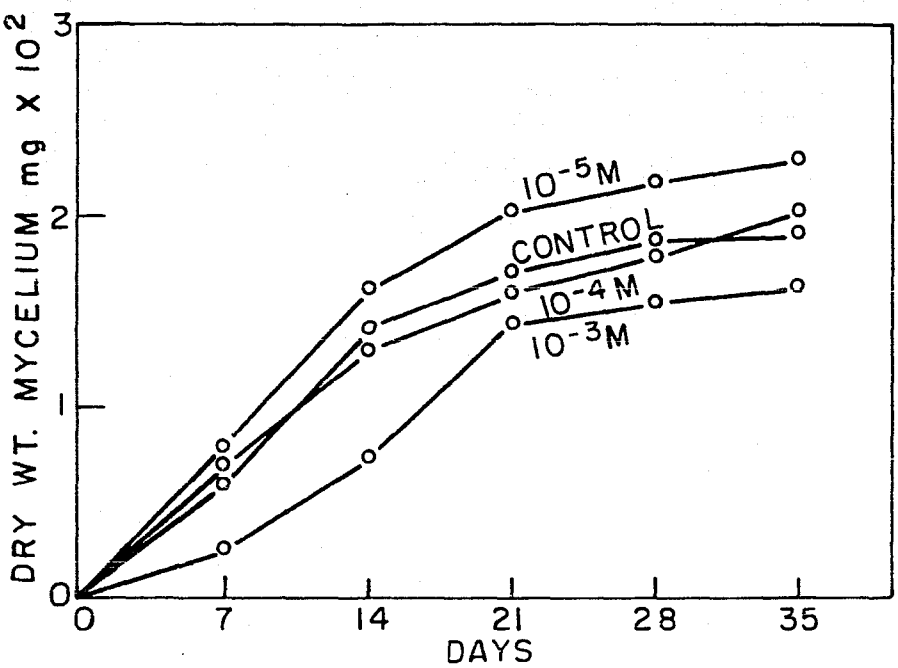

DRY WT. MYCELIUM mg X 10²
3
2
1
0
0
7
14
21
28
35
DAYS
CONTROL
10⁻⁵M
10⁻⁴M
10⁻³M

Figure 6. Growth of E. fagacearum as affected by quercitin

Figure 7. Growth of E. fagacearum as affected by resorcinol


DRY WT. MYCELIUM mg X 10²
3
2
1
0
10⁻⁴ M
10⁻⁵M
CONTROL
10⁻³M
0
7
14
21
28
35
DAYS


DRY WT. MYCELIUM mg X 10²
3
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CONTROL
10⁻⁴ M
10⁻³M
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7
14
21
28
35
DAYS

test this hypothesis, culture filtrates and spore suspensions of E. fagacearum were prepared and injected into red oak. Neither the culture filtrates nor the spore suspensions had any effect on the rate of formation of tyloses (Table 18).

Table 18. Tylosis formation as affected by culture filtrates and spore suspensions of E. fagarearum

Treatment	R value
Culture filtrates	
one week old	0.23
two weeks old	0.22
three weeks old	0.24
two weeks old (concentrated 1/2)	0.21
three weeks old (concentrated 1/2)	0.22
Spore suspensions	
in distilled water	0.21
in basal medium	0.22
Controls	
in distilled water	0.23
in basal medium	0.22

In an attempt to isolate an enzyme fraction from the culture filtrate 200 ml fractions of 15 day old culture filtrates were evaporated rapidly at 30°C to 50 ml and immediately chilled in an ice bath. These concentrated fractions were adjusted from pH 2.0 to 12.0 by addition of 1.0 N NaOH or 1.0 N HCl. Sufficient chilled acetone was added to the fractions to bring the final concentration to 50 per cent. The solutions were stirred rapidly whereupon the polysac-

charide component floated to the top and was removed. The remaining colorless precipitates in the solutions were filtered and redissolved in 10.0 ml of citric acid buffer pH 6.0. These precipitates had no stimulatory effect on tylosis formation (Table 19). Other precipitates obtained in the same manner were collected by filtration, tested for the

Table 19. Tylosis formation as affected by solutions of the 50 per cent acetone precipitates from culture filtrates of E. fagacearum

	pH	mg precipitate	R value
Tree 1	2.0	21.53	0.28
	3.0	24.78	0.31
	4.0	24.01	0.27
	5.0	24.89	0.27
	6.0	35.31	
Control			0.27
Tree 2	7.0	41.46	0.31
	8.0	40.09	0.29
	9.0	43.21	0.30
	10.0	50.61	0.31
	11.0	53.25	0.30
Control			0.31

presence of peptide bonds with the biuret reagent, dried and weighed (Table 19). The results of the biuret test were negative and therefore the precipitates did not contain a protein moiety.

Presence of growth promoting substances in culture filtrates

Presence of growth promoting substances in culture filtrates of E. fagacearum was investigated on the assumption that such substances might have some role in tylosis formation. Avena coleoptile tests on culture filtrates of different ages were undertaken. Pronounced elongation of coleoptile sections was observed for filtrates from 11- to 15-day-old cultures (Table 20). Beyond this time toxic substances apparently were present which interfered with the test.

Table 20. Growth of Avena coleoptile sections as affected by culture filtrates

Age of culture in days	Weight of mycelium in mg	pH	Length of coleoptiles	Standard deviation
0 (control)			5.35	0.24
10	25.21	6.2	5.08	0.30
11	32.57	6.3	5.70	0.32
12	42.41	6.1	5.83	0.29
13	56.71	6.0	6.37	0.29
14	68.01	6.1	5.85	0.19
15	75.73	5.6	5.74	0.21
16	84.30	5.6	4.85	0.26

Attempts were made to isolate the substance or substances inducing the stimulation. A 200 ml sample of the 13-day-old culture filtrate concentrated (to 20 ml) at 32°C *in vacuo* was extracted with peroxide free diethyl ether overnight in a continuous extractor. The ether layer was removed, evaporated *in vacuo* and the ether soluble components taken up in 1.0 ml of 50 per cent ethanol. From this solution 200 µl aliquots were applied to chromatograms developed in 80 per cent phenol. After development the chromatograms were thoroughly dried and sprayed with the indole reagent (Block *et al*. 1955). No indole positive spots were observed. To be certain the extraction procedure was not at fault a 100 µg sample of indole acetic acid was dissolved in 200 ml of culture filtrate and the identical extraction procedure carried out. On the basis of area measurement, an 82 per cent recovery of indole acetic acid was estimated. With this information it appeared that ether soluble indole compounds were not present in culture filtrates.

Since some amino acids, in addition to specific growth regulators, promote elongation of coleoptiles (Nitsch and Nitsch 1956) an attempt was made to determine the amino acids present in 13-day-old culture filtrates. The presence of l-arginine in relatively high concentration in the basal medium prevented the direct chromatographic determination of other amino acids. To circumvent this, a 50 ml sample of the 13-

day-old culture filtrate was concentrated *in vacuo* at 32°C and passed through a Dowex 50 x 8 cation exchange column. After elution the cation fraction was concentrated to dryness *in vacuo* at 32°C and taken up in 1.0 ml of distilled water. This solution was banded on two descending chromatograms developed in 80 per cent phenol. After drying the chromatograms were cut into strips corresponding to Rf values from 0.1 to 1.0 and eluted in distilled water. Eluates were diluted to 2.0 ml and each was divided in half. One half of each eluate was applied to a chromatogram in varying concentrations. After development in BABW chromatograms were sprayed with ninhydrin and amino acids marked. From a previously prepared chromatomap tentative identification of the amino acids was made. Varying concentrations of the other half of each eluate were then chromatographed against known amino acids selected on the basis of prior tentative identification. It was necessary to carry some eluates through another chromatographic separation to resolve those amino acids having Rf values close to arginine in both 80 per cent phenol and BABW. By this method identical Rf values and reactions with ninhydrin were recorded for the following amino acids: lysine, tyrosine, methionine, threonine and glycine. Since methionine specifically stimulated the growth of coleoptile sections in the absence of IAA (Nitsch and Nitsch 1956), it was concluded that this substance, in all probabil-

ity, accounted for the observed stimulation of coleoptiles by culture filtrates of E. fagacearum.

The rather abrupt termination of the growth-promoting characteristics of culture filtrates was difficult to understand. Sugar concentrations above 2.0 per cent adversely affected coleoptile elongation. However, although the molar concentration of sugar probably increased in the medium due to hydrolysis of sucrose to glucose and fructose, the sugar concentration in the test solutions never exceeded the 2.0 per cent optimum (Nitsch and Nitsch 1956). A study of the mycelial weights (Table 20 and Figure 8) indicated that the organism was in the phase of maximum growth. At this time there presumably would be a rapid increase in extra-cellular metabolic products, any one of which might interfere with the growth-promoting effects of methionine.

Toxicity of culture filtrates to red oak leaves

Evidence points to nutritional deficiencies rather than toxins, as the primary cause of leaf symptoms on diseased red oak. A preliminary study of basic metabolic products of the organism was attempted however in hope that some information might be obtained leading to a more fundamental understanding of the pathogenicity of E. fagacearum.

Culture filtrates of E. fagacearum grown under various conditions, have been shown to contain materials toxic to

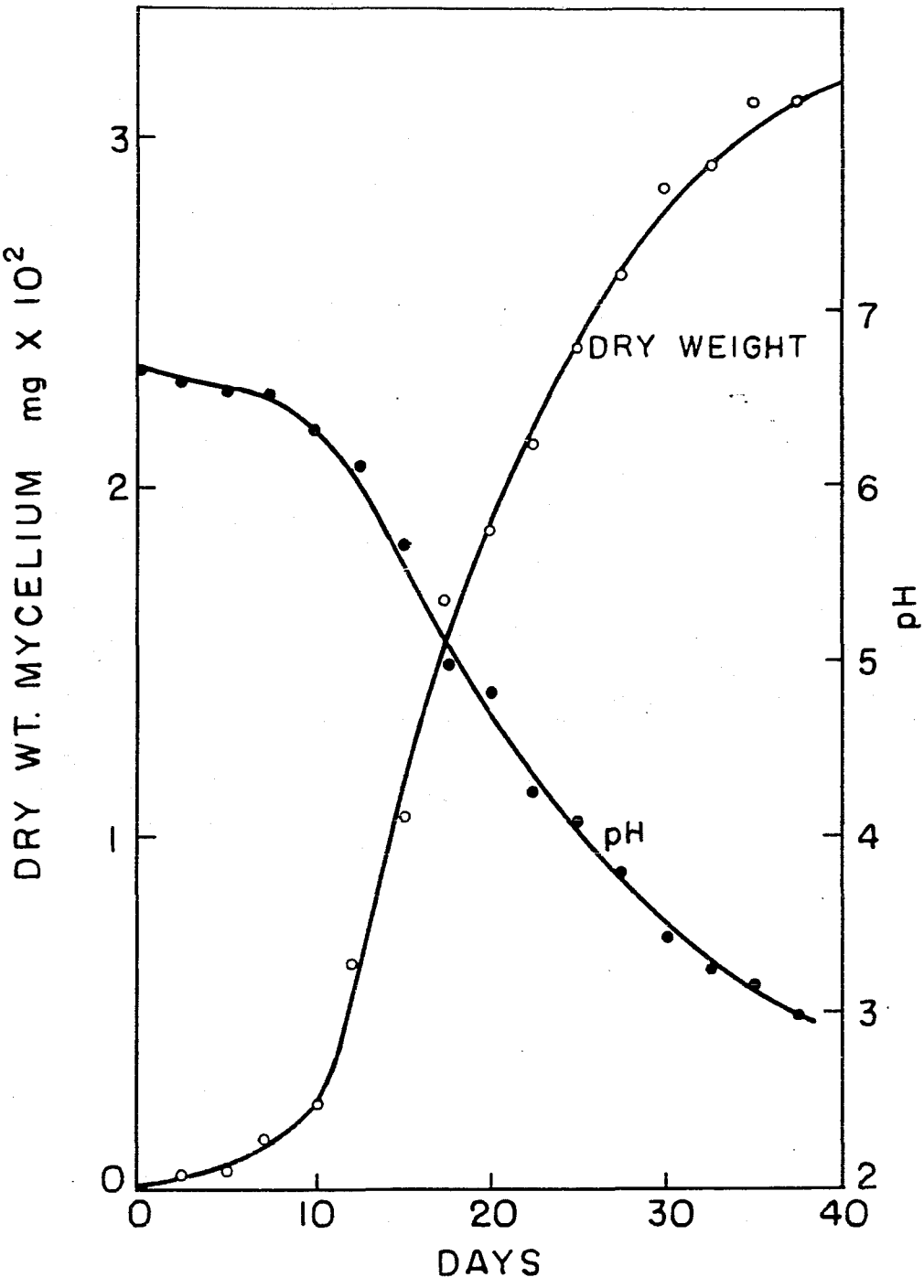
red oak leaves and tomato cuttings (White 1955; Hoffman 1954). The toxic substances could be partially separated and purified by precipitation and extraction procedures.

As a preliminary step, one- to four-week-old culture filtrates, with no preliminary treatment, were tested for toxicity to red oak leaves. A growth curve of the organism (Figure 8) was prepared in conjunction with this phase of the study. While wilting of red oak leaves resulted when the leaves were placed in the filtrates of two- to four-week-old cultures there was no evidence of toxic substances inducing leaf necrosis. When a polysaccharide-like material was removed by precipitation with 95 per cent ethanol and the ethanol removed *in vacuo*, the culture filtrates were not toxic within the limits of the experiment.

Fractionation of culture filtrates

The alcohol insoluble fraction. *E. fagacearum* produced in culture a gelatinous polysaccharide-like material. Four 20-day-old cultures of the organism grown in the basal medium were filtered and the filtrate concentrated *in vacuo* to 30 ml. Enough 95 per cent ethanol was added to bring the alcohol concentration to 50 per cent. This was stored for several hours at 0°C after which the gelatinous, grey polysaccharide material was collected on a filter paper and washed with warm 95 per cent ethanol. It was redissolved in warm water

Figure 8. Growth of E. fagacearum in the basal medium


DRY WT. MYCELIUM mg X 10^2
DRY WEIGHT
pH
DAYS
0
1
2
3
0
10
20
30
40
2
3
4
5
6
7

again precipitated with ethanol and filtered. This was repeated until the gelatinous material appeared completely homogenous after which it was re-dissolved in 3.0 ml hot water and an equal volume of 3.0 N H_2SO_4 added. The solution was heated for three hours under reflux on a steam bath, cooled, saturated with $BaCO_3$, and the excess $BaCO_3$ and $BaSO_4$ removed by filtration. The solution was then evaporated to dryness and taken up in 1.0 ml of hot pyridine. This material was applied to chromatograms and developed for 70 hours by the descending method in BABW. After drying the chromatograms were sprayed with AHP and heated for five minutes at 100°C. One spot was observed which proved identical to glucose by co-chromatography with an authentic sample. Therefore, under conditions where sucrose was used as a carbon source a polysaccharide-like material was isolated containing glucose as the only reducing sugar. The possibility existed that the polysaccharide was a glucosan.

To determine the nature of the polysaccharide when other sugars were used as a carbon source, the basal medium was modified to incorporate 0.1 M fructose, l(+) arabinose or l-xylose in place of sucrose. At the end of 20 days the cultures were treated in the same manner as indicated previously, to isolate the polysaccharide. Under the conditions where arabinose and xylose were used as the carbon source only one reducing substance corresponding to glucose

was isolated. The polysaccharide observed when fructose was used as the carbon source appeared to contain a small quantity of fructose, in addition to glucose. In this case either a heterogeneous polysaccharide or another polysaccharide distinct from the one containing glucose was present.

To determine if the polysaccharide produced by E. fagacearum on a sucrose substrate would induce wilting in oak leaves the polysaccharide was purified from 100 ml of culture filtrate dissolved in 5 ml of distilled water and its effect on the transpiration of oak leaves measured (Figure 8). To do this the petiole of each leaf was inserted into a small vial containing distilled water. The tops of the vials were sealed so that loss of water occurred only from the leaf surface. The vial was placed on a balance and weighed every five minutes over a short period to obtain an estimation of the normal transpiration rate. After equilibration for 40 minutes, all but 0.1 ml of the water was removed from the vial with a hypodermic syringe and replaced with 1.0 ml of the polysaccharide solution. The polysaccharide at this concentration induced an immediate response reflected in the rapid decrease in transpiration. Within a 12 hour period the leaves in the polysaccharide solution had wilted completely. When a similar experiment was carried out using a washed spore suspension (three times in double-distilled water) of E. fagacearum containing approximately 2.5×10^4

spores/ml in place of the polysaccharide the response was similar (Figure 9). These results were typical of the action of large molecules or spores on the transpiration rate of leaves (Gäumann 1951).

The ether soluble fraction. To 200 ml of the culture filtrate concentrated *in vacuo* to 60 ml, 50 ml of peroxide free diethyl ether was added. The mixture was shaken thoroughly for five minutes and held in the dark at about 20°C for one week. The yellow ether layer was then removed and evaporated *in vacuo* to dryness. A yellow wax-like residue remained. The ether soluble fraction was shaken with 10 ml of distilled water and filtered. This aqueous solution, which fluoresced bright blue under UV, exhibited no toxicity to oak leaves. Most of the yellow wax-like material was insoluble in distilled water. The residue was taken up in 2.0 ml of 95 per cent ethanol in which it was readily soluble. The solution was banded on a chromatogram and developed in BAW. After drying, the compound which had an Rf of 0.96 in this solvent could be seen as a very faint yellow band in visible light and as a bright blue fluorescent band under UV. It exhibited no color change in the presence of ammonia fumes. The fluorescent band was eluted from the chromatogram with 95 per cent ethanol and its UV spectrum (Figure 10) determined with the Beckman DU spectrophotometer. At this concentration the substance did not appear to have any absorption

Figure 9. Decline in the transpiration rate of red oak leaves in a spore suspension of E. fagacearum and a solution of the polysaccharide

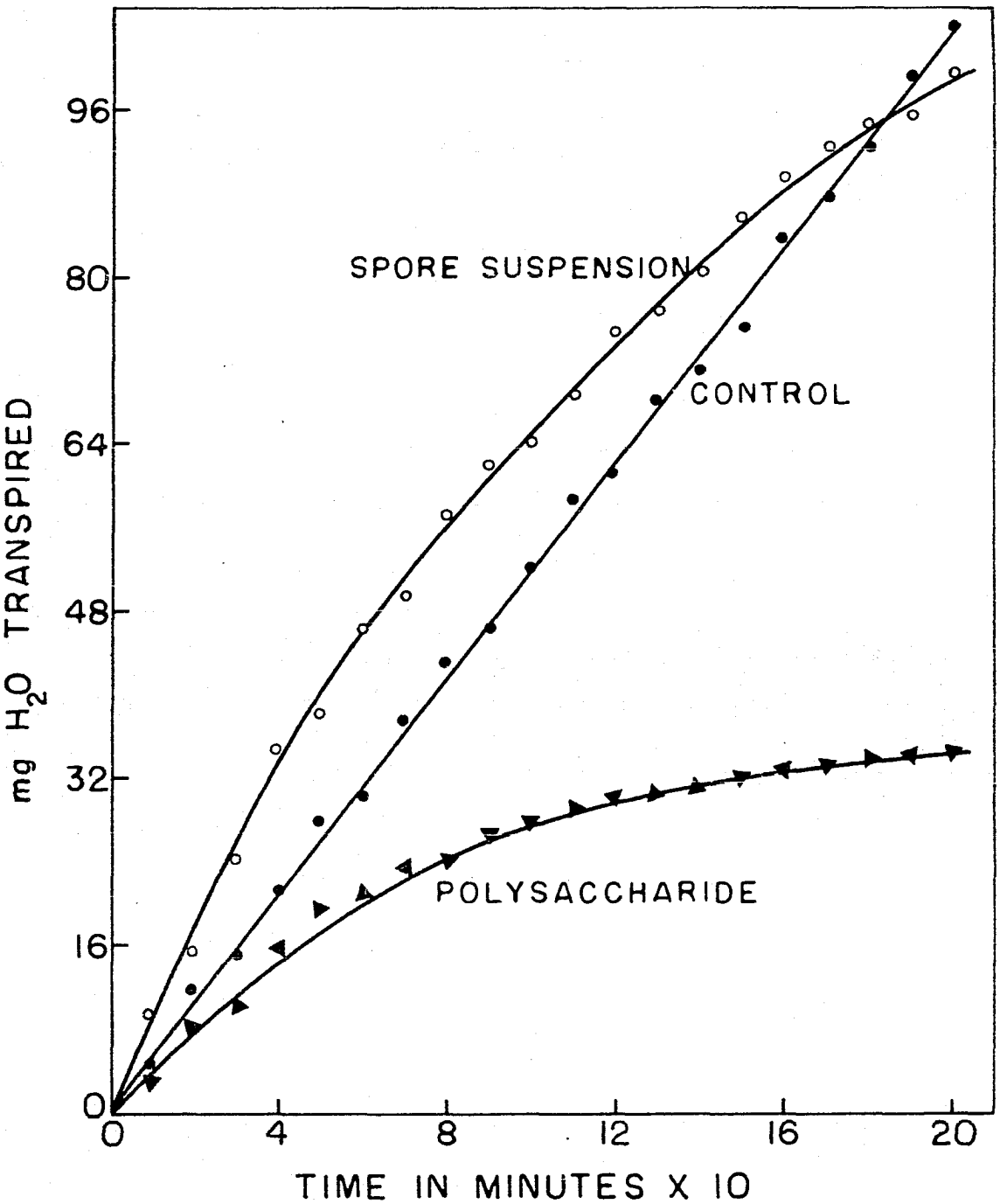
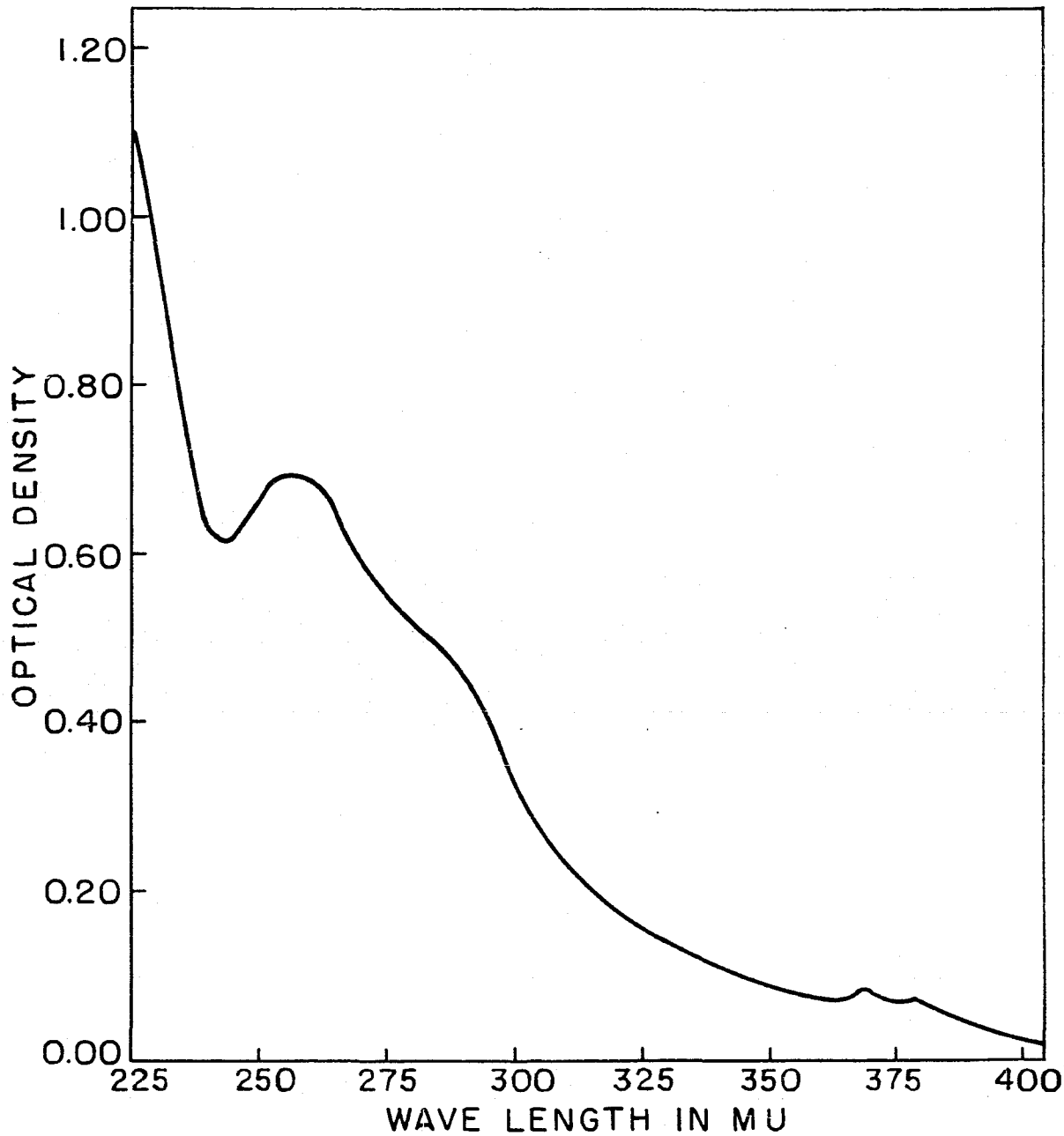

96
80
64
48
32
16
0
mg H_2O TRANSPIRED
SPORE SUSPENSION
CONTROL
POLYSACCHARIDE
0
4
8
12
16
20
TIME IN MINUTES X 10

Figure 10. The UV absorption spectrum of a substance obtained from culture filtrates of *E. fagacearum*


1.20
1.00
0.80
0.60
0.40
0.20
0.00
OPTICAL DENSITY
225
250
275
300
325
350
375
400
WAVE LENGTH IN MU

maxima in the visible range.

The cation fraction. Exactly 200 ml of the culture filtrate were evaporated down to 50 ml in vacuo at 36°C. The filtrate was mixed with 55 ml of 95 per cent ethanol and the precipitate removed by filtration. The remaining solution was evaporated down to 20 ml and passed through a Dowex 50 x 8 cation exchange resin. This procedure was identical to that described previously. The cation fraction was concentrated in vacuo to 1.0 ml, spotted on several chromatograms developed two dimensionally in 80 per cent phenol and BABW. The ninhydrin positive areas were located and outlined on duplicate chromatograms. After drying the chromatograms in a warm oven until all the odor of phenol had disappeared they were eluted. Various dilutions of these eluates were examined for toxicity to soy bean seedlings, variety Clark, in the unifoliate stage, since oak leaves were no longer available. The eluates were toxic to soybeans causing interveinal browning followed by rapid death of the leaf tissue. Duplicate eluates were banded on descending chromatograms to resolve the toxic component. Since the eluates from these chromatograms proved to be uniformly toxic it was assumed that some substance in the solvent employed was responsible. A further study revealed that phenol at dilutions down to one part in 1×10^6 parts of distilled water induced an identical response. Subsequent

analysis of the cation fraction identified the amino acids as those previously found in 13-day-old culture filtrates. These chromatographically identified amino acids were not tested for toxicity to soybeans.

Isolation of toxic substances by steam distillation. A 200 ml quantity of the culture filtrate was steam distilled at atmospheric pressure. The distillate was collected in 10 ml aliquots until 70 ml had been distilled. The seven fractions were then tested for toxicity to soybean seedlings. The first 10 ml fraction induced marginal necrosis and shrivelling of the leaf tissue within 24 hours. An attempt was made to determine the volatile components of this fraction.

Identification of possible volatile organic acids was undertaken by the preparation of their hydroxamates. Four 20 ml aliquots of the first fraction (pH 5.5) were neutralized with 1.0 N NaOH and evaporated to dryness in vacuo. To the residue was added 5.0 ml of methanol containing 0.15 ml of concentrated H_2SO_4. The solution was held for 24 hours at 36°C in a sealed weighing bottle after which equal volumes of 5.0 per cent hydroxylamine-HCl in methanol and 12.5 per cent NaOH in methanol were added. The solution was heated to boiling under reflux on a steam bath, cooled, left for one hour, and neutralized to litmus with methanolic H_2SO_4. This solution was filtered, evaporated to 0.5 ml and

a small portion tested for the presence of hydroxamates with $FeCl_3$. A faint positive reaction was observed, which corresponded to the Rf values of the hydroxamate of acetic acid prepared in an identical manner from sodium acetate.

To prepare the 2,5,dinitrophenylhydrazones 20 ml of the first fraction were mixed with twice their volume of a saturated solution of 2,4,dinitrophenylhydrazine in 20 N HCl. The solution was placed in a boiling water bath for five minutes whereupon a precipitate formed. After cooling, the solution was filtered, the precipitate washed with distilled water and dried. The filtrate was extracted with small portions of ethyl acetate. The ethyl acetate solution was concentrated and chromatographed in ethyl alcohol:petroleum ether (Skelly B), 80:20 v/v (Block et al. 1955). Three yellow spots were observed. Their Rf values were 0.96, 0.75, and 0.62, respectively. The substance at Rf 0.96 proved chromatographically identical to the 2,4,dinitrophenylhydrazone of acetaldehyde, the substance at Rf 0.62 was unreacted 2,4,dinitrophenylhydrazine. The identity of the substance corresponding to Rf 0.75 was not known. The crystalline hydrazone on chromatography in several solvents acted identically to the 2,4,dintrophenylhydrazone of acetaldehyde.

The steam distillate of the culture filtrate which prior to distillation had been made alkaline with 2N NaOH proved to be equally as toxic as the steam distilled fraction from

untreated culture filtrates. From this information it was assumed that the volatile acid fraction was probably not responsible for the observed toxicity of the first fraction to soybean seedlings. Acetaldehyde in concentrations exceeding one part in 10^4 parts of distilled water caused complete collapse of the leaves and stems of the seedlings. At concentrations lower than this, down to one part in 10^6 parts of distilled water, marginal necrotic spots developed and general yellowing of the leaf tissue appeared in two to five days. However the symptoms induced by acetaldehyde were the same as those observed in control seedlings after approximately 10 days in distilled water. These symptoms, typical of potassium deficiency, that the presence of a common metabolic intermediate under unbalanced nutritional conditions hastened their appearance. Since these symptoms were only superficially similar to those observed on leaves of soybean seedlings placed in the steam distilled fraction, the toxic substance in the steam distillate was assumed not to be acetaldehyde.

Other metabolites from culture filtrates. Determination of specific products of metabolism in mycelial extracts or culture filtrates is useful in the elucidation of metabolic pathways of organisms. Once established, possible toxic substances can be deduced or hypothesized and precise methods undertaken to isolate them. In anaerobic culture ethyl

alcohol is often the first product of metabolism accumulated in excess. Since ethanol is a known toxin (Gäumann 1957) an attempt was made to determine the quantity of ethanol produced in still culture by this pathogen.

A standard curve was prepared from 0.0 to 5.0 per cent ethanol at 15°C with a Cenco specific gravity hydrometer (0.900 to 1.100). From 15-day-old culture filtrates a 200 ml sample was distilled at atmospheric pressure until 30 ml of distillate had been collected. The specific gravity of this solution at 15°C exceeded 1.000 indicating the presence of contaminating substances. Consequently 200 ml of the culture filtrate were treated with 6.0 N NaOh for 40 minutes to saponify any esters present, and when cool, with dilute ASN to oxidize volatile aldehydes and ketones. Distillation of 30 ml of this solution yielded a distillate with specific gravity of about 1.000. As a consequence it was assumed that the metabolism of E. fagacearum did not result in the accumulation of ethanol. Subsequently, the distillate was redistilled and the xanthogenate derivatives prepared (Block et al. 1955). On chromatography in n-butanol saturated with 5.0 per cent $NaHCO_3$ only a trace of ethyl xanthogenate was observed (Rf 0.28) indicating that ethanol was probably a transient in metabolism, not accumulated in any quantity. An indication that metabolism might proceed via ethanol was observed during initial studies of the mineral requirements

of the organism. Growth, after 40 days in the basal medium from which zinc had been deleted, averaged 92.6 mg. From the corresponding controls the average was 363.0 mg. While zinc undoubtedly has other important roles in metabolism it is a co-factor requirement for alcohol dehydrogenase.

To establish the presence of keto acids in the culture filtrate, a 25 ml sample of the filtrate was chilled in an ice bath and mixed with 50 ml of a cold saturated solution of 2,4,dinitrophenylhydrazine in 2.0 N HCl. The solution was held at 36°C for 30 minutes and then extracted with several small portions of ethyl acetate. The ethyl acetate was in turn extracted with 1.0 N ammonium carbonate. The alkaline solution was extracted twice with ethyl acetate, chilled, acidified with cold 2.0 N HCl and re-extracted with ethyl acetate. The ethyl acetate fraction was evaporated to dryness and dissolved in a minimum of distilled water. The solvent n-butanol: ethanol: 0.5 N NH_4OH(Aronoff 1956), proved best for the resolution of the keto acid-2,4,dinitrophenylhydrazones. By chromatography with authentic samples prepared in an identical manner, pyruvic acid-2,4,dinitrophenylhydrazone (Rf 0.43) and ∝ keto-glutamic acid-2,4,dinitrophenylhydrazone (Rf 0.19) were identified. An unidentified substance (Rf 0.75) in relatively higher concentration was also observed. It was not the 2,4,dinitrophenylhydrazone of oxalacetate or unreacted 2,4,dinitrophenylhydrazine. While

no attempt was made to characterize fully this substance the Rf value (0.62) in tert-amyl alcohol: ethanol: distilled water, 50:10:40 v/v (Block et al. 1955) suggested it might be p-hydroxyphenylpyruvic acid.

GENERAL DISCUSSION AND CONCLUSIONS

The experimental work presented herein was undertaken in the hope of clarifying some biochemical aspects of host-parasite relationships in oak wilt. Both leaf symptoms and tylosis formation, a pronounced vascular symptom of this disease, were studied. While these investigations have not resulted in an explanation of the fundamental causes of all the complex symptom developments in oak wilt, some of the more basic aspects have been resolved. It was concluded that the role of E. fagacearum in the wilt syndrome cannot be interpreted fully on the basis of the toxin theory.

Chromatographic and analytical evidence, in addition to other observations, pointed to nutrient deficiencies as the primary cause of necrotic and chlorotic leaf symptoms. These diseased leaves in contrast to healthy leaves exhibited deficiencies of total nitrogen, phosphorus, soluble carbohydrates and free amino acids. Starch accumulation, common when nutrients or moisture are deficient, also occurred in diseased leaves. During periods of maximum growth oak leaves appeared to have a marked tolerance in regard to water requirements. By a drought hardening process, achieved perhaps through dehydration of the protoplasm, leaves were able to survive a severe water shortage. Specific nutrient demands for growth and maintenance probably are not so regulated and therefore, death or chlorosis of tissue eventually re-

sulted.

Studies on wounded and drought-killed red oak suggested that cessation of xylem sap flow played a prominent role in tylosis formation. Tyloses occurred extensively in conducting vessels of red oak dying from drought injury. After wounding, the horizontal, radial and vertical distribution of tyloses was confined to areas through which xylem sap flow had been obstructed by the wound. Why tylosis formation resulted from cessation of xylem sap flow was not explained unequivocally. Most of the evidence did not support existence of a specific hormone-like substance which could induce tyloses. Various chemical fractions isolated from sapwood and separated by chromatographic means induced no observable stimulation to tylosis formation. Materials prepared from culture filtrates and spore suspensions of E. fagacearum also did not alter the rate of tylosis formation. Therefore, it is suggested that when cessation of xylem sap flow is induced by drought, wounding, senesence or mechanical plugging metabolic processes of the host are re-directed and tyloses result.

Chromatographic identification of aesculin in the xylem of diseased oak is of interest in light of the known role of coumarin and coumarin derivatives as auxin inhibitors. By use of standard techniques, investigation of auxin and anti-auxin relationships might prove valuable. The

remaining identified or partially identified compounds appeared to be common phenolic or flavonoid constituents of the host. Their role, if any, in tylosis formation remains to be determined.

The presence of toxic substances, polysaccharides and growth stimulants in culture filtrates of E. fagacearum was demonstrated. The polysaccharide-like material contained glucose and was shown to cause wilting of red oak leaves. Of the fractions assayed for phytotoxic activity only the steam distillate of culture filtrates was toxic. Studies on growth stimulants produced in culture failed to reveal any evidence of indole-type compounds. Chromatographic analyses of amino acids and other constituents present in culture filtrates suggested that the amino acids and in particular methionine were responsible for the observed stimulation of Avena coleoptile sections. Previously derived conclusions on the causes of leaf symptoms do not implicate fungus-synthesized toxins. However, polysaccharides synthesized by the fungus in addition to various products of host-parasite interaction could be involved as physical agents in the plugging mechanism.

LITERATURE CITED

Aronoff, Sam. 1956. Techniques of radiobiochemistry. Ames, Iowa, Iowa State College Press.

Beckman, C. H., J. E. Kuntz and A. J. Riker. 1953a. The growth of the oak wilt fungus with various vitamins and carbon and nitrogen sources. Phytopath. 43:441-447.

_____, _____, _____ and J. G. Berbee. 1953b. Host responses associated with oak wilt development. Phytopath. 43:448-454.

Bewley, W. F. 1922. "Sleepy disease" of the tomato. Ann. Appl. Biol. 9:116-134.

Block, R. 1952. Wound healing in higher plants 2. Bot. Rev. 18:655-682.

Block, R. J., B. L. Durrum and G. Zweig. 1955. A manual of paper chromatography and paper electrophoresis. New York, N. Y., Academic Press, Inc.

Bollard, E. G. 1953. Movement of nitrogenous substances of apple tree sap. Nature (London) 171:671-672.

Bonner, J. and J. English, Jr. 1938. A chemical and physiological study of traumatin a plant wound hormone. Plant Physiol. 13:331-348.

Brandes, E. W. 1919. Banana wilt. Phytopath. 9:339-389.

Brown, A. B. and R. G. H. Cormack. 1937. Stimulation of cambium activity, locally in the region of application and at a distance in relation to a wound, by means of heteroauxin. Canad. Jour. Res. 15:433-441.

Burrows, S., F. S. M. Grylls and J. S. Harrison. 1952. Paper chromatography of phosphoric esters. Nature (London) 170:800-801.

Chattaway, Margaret M. 1949. The development of tyloses and secretion of gum in heartwood formation. Austral. Jour. Sci. Res. (B) 2:224-240.

Chrysler, M. A. 1908. Tyloses in tracheids of conifers. New Phytol. 7:198-204.

Clarke, D. D. and F. F. Nord. 1955. Simple benzene derivatives. In Paech, K. and H. V. Tracey, eds. Moderne Methoden der Pflanzenanalyse. Vol. 3, pp. 332-360. Berlin, Germany, Springer.

Clinton, G. P. and Florence A. McCormick. 1936. Dutch elm disease. Conn. (New Haven) Agr. Exp. Sta. Bul. 389: 701-752.

Consden, R. 1948. Partition chromatography on paper, its scope and application. Nature (London) 162:359-361.

Cook, A. H., S. F. Cox, T. H. Farmer and M. S. Lacey. 1947. Production of antibictics by Fusaria. Nature (London) 160:31-32.

Davis, E. A. 1949. Effects of several plant growth regulators on wound healing of sugar maple. Bot. Gaz. 111: 69-77.

Davis, David and A. E. Dimond. 1954. The source and role of phenols in Fusarium wilt symptoms. Phytopath. 44: 485-486.

_____ and P. E. Waggoner. 1953. Conjugated phenols in the Fusarium wilt syndrome. Nature (London) 172:959-961.

Dimond, A. E. 1955. Pathogenesis in the wilt diseases. Ann. Rev. Plant Physiol. 6:329-350.

_____ and P. E. Waggoner. 1953a. On the nature and role of vivotoxins in plant disease. Phytopath. 43:229-235.

_____ and _____ 1953b. The cause of epinastic symptoms in Fusarium wilt of tomatoes. Phytopath. 43:663-669.

_____, G. H. Plumb, E. M. Stoddard and J. G. Horsfall. 1949. An evaluation of chemotherapy and vector control by insecticides for controlling Dutch elm disease. Conn. (New Haven) Agr. Exp. Sta. Bul. 531:1-69.

Engelhard, A. W. 1955. Host parasite relationships of Endoconidiophora fagacearum Bretz, the cause of oak wilt. Unpublished Ph. D. Thesis. Ames, Iowa, Iowa State College Library.

_____ and W. H. Bragonier. 1957. Dwarf leaf, a symptom of oak wilt. (Abstract) Phytopath. 47:10.

English, J. Jr., J. Bonner and A. J. Haagen Smit. 1939. Structure and synthesis of a plant wound hormone. Science 90:329.

Esau, Katherine. 1949. Anatomic effects of the viruses of Pierce's disease and phony peach. Hilgardia 18:423-482.

Fergus, C. L. and D. C. Wharton. 1957. Production of pectinase and growth promoting substances by Ceratocystis fagacearum. Phytopath. 47:635-636.

Gäumann, E. 1951. Some problems of pathological wilting in plants. Adv. Enzym. 11:401-437.

_____ 1957. Fusaric acid as a wilt toxin. Phytopath. 47:342-357.

_____, St. Roth, L. Ettlinger, P. A. Plattner and U. Nager. 1947. Enniatin, ein neves, gegen Mykobakterien wirksames Antibioticum. Experientia 3:202.

Geissman, T. A. 1955. Anthocyanins, chalcones, aurones, flavones and related water soluble plant pigments. In Paech, K. and M. V. Tracey, eds. Moderne Methoden der Pflanzenanalyze. Vol. 3, pp. 450-498.

Gerry, Eloise. 1914. Tyloses: their occurrence and practical significance in some American woods. Jour. Agr. Res. (U.S.) 1:445-469.

Goodwin, R. H. and Kavanaugh. 1949. The isolation of scopoletin, a blue fluorescing compound from oat roots. Bul. Torrey Bot. Club 76:255-265.

Gore, D. N. 1951. The detection of acids on paper partition chromatograms. Chem. and Ind. 4:479.

Gothoskar, S. S., R. P. Scheffer, J. C. Walker and M. A. Stahmann. 1953. The role of pectic enzymes in Fusarium wilt of tomato. Phytopath. 43:535-536.

Haberlandt, G. 1921. Wundhormone als erreger von zellteilungen. Beitr. Allg. Bot. 2:1-53.

Hammet, F. S. and S. S. Chapman. 1938. Correlation between sulf hydrymitosis and cell growth in length of roots of Phaseolus vulgaris. Growth 2:297-302.

Hanes, C. S. 1940. The reversible formation of starch from glucose-1-phosphate catalyzed by potato phosphorylase. Proc. Roy. Soc. (London)(B) 129:174-208.

Hodgson, R., W. H. Peterson and A. J. Riker. 1949. The toxicity of polysaccharides and other large molecules to tomato cuttings. Phytopath. 30:47-62.

Hoffman, P. F. 1954. Physiology of Endoconidiophora fagacearum Bretz, 1. Factors influencing growth and toxin production. Iowa State Coll. Jour. Sci. 29:27-38.

Hutchinson, C. H. 1913. Rangpur tobacco wilt. Memoirs India Agr. Dept., Bact. Series 1:67-84.

Klein, G. 1923. Zur Atiologie der Thyllen. Zeitschrift fur Botanik 15:418-439.

Küster, E. 1925. Pathologische pflanzenanatomie. 2nd ed. Jena, Germany, Gustav Fisher.

LaRue, C. D. 1941. The effect of wounding and wound hormones on root formation. Proc. Nat. Acad. Sci. 27: 388-392.

Maizel, J. W., A. A. Benson and N. E. Tolbert. 1956. Identification of phosphoryl choline as an important constituent of plant saps. Plant Physiol. 31:407-408.

Nitsch, J. P. and Collette Nitsch. 1956. Studies on the growth of coleoptile and first internode sections. A new sensitive, straight-growth test for auxins. Plant Physiol. 31:94-111.

Powers, H. R., Jr. 1954. The mechanism of wilting in tobacco plants affected by black shank. Phytopath. 44:513-521.

Roberts, E. A. H. and D. J. Wood. 1951. A study of polyphenols in tea leaf by paper chromatography. Biochem. Jour. 49:414-422.

Rosen, H. R. 1926. Efforts to determine the means by which the cotton wilt fungus, Fusarium vasinfectum induces wilting. Jour. Agr. Res. 33:1143-1163.

Rudolph, E. E. 1922. Verticillium hadromycosis. Hilgardia 5:201-361.

Sleeth, B. 1933. Relationship of Fusarium niveum to the formation of tyloses in melon plants. (Abstract) Phytopath. 23:33.

Struckmyer, Esther B., C. H. Beckman, J. E. Kuntz and A. J. Riker. 1954. Plugging of vessels by tyloses and gums in wilting oaks. Phytopath. 44:148-153.

Sumner, J. B., T. C. Chou and A. T. Bever. 1950. Phosphorylase of jack bean. Arch. Biochem. 26:1-5.

Swain, T. 1953. The identification of coumarins and related compounds by filter paper chromatography. Biochem. Jour. 53:200-208.

van der Meer, J. H. H. 1926. Verticillium wilt of maple and elm seedlings in Holland. Phytopath. 16:611-614.

Waggoner, P. E. and A. E. Dimond. 1955. Production and role of extracellular pectic enzymes of Fusarium oxysporum f. lycopersici. Phytopath. 45:79-87.

Ward, G. H. and F. B. Johnston. 1953. Chemical methods of plant analysis. Canada Dept. of Agr., Chemistry Div. Contrib. No. 238.

White, Irene G. 1955. Toxin production by the oak wilt fungus Endoconidiophora fagacearum Bretz. Amer. Jour. Bot. 42:759-764.

Widdowson, E. M. 1932. Physiology of apples. 13. Starch and hemicellulose contents of developing apples. Ann. Bot. 46:597-631.

Williams, S. 1942. Secondary vascular tissues of the oaks indigenous to the U. S. 2. Types of tyloses and their distribution in Erythobalanus and Leucobalanus. Bul. Torrey Bot. Club 69:1-10.

Winstead, N. N. and J. C. Walker. 1954. Production of vascular browning by metabolites from several pathogens. Phytopath. 44:153-158.

Yabuta, T., K. Kambe and T. Hayashi. 1934. Biochemistry of the bakanae fungus. I. Fusarinic acid a new product of the bakanae fungus. Jour. Agr. Chem. Soc. Japan 10:1059-1068.

Young, Roy Alton. 1949. Studies on oak wilt caused by Chalara quercina. Phytopath. 39:425-441.

Zentmyer, G. A. 1942. Toxin formation by Ceratostomella ulmi. Science 95:512-513.

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