Factors Influencing Asexual Spore Formation by Ceratocystis ULMI

Richard J. Roster

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FACTORS INFLUENCING ASEXUAL SPORE FORMATION

BY CERATOCYSTIS ULMII

BY

RICHARD J. ROSTER

A thesis submitted
in partial fulfillment of the requirements for the
degree Master of Science, Major in
Botany, South Dakota
State University

1970
FACTORS INFLUENCING ASEXUAL SPORE FORMATION

BY CERATOCYSTIS ULCI

This thesis is approved as a creditable and independent investigation by a candidate for the degree, Master of Science, and is acceptable as meeting the thesis requirements for this degree, but without implying that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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ACKNOWLEDGMENT

I would like to take this opportunity to thank Dr. David J. Holden for his suggestions, guidance and encouragement throughout this research. I also wish to express my appreciation to all members of the Botany-Biology Department for their assistance and interest in this project. A special thanks to Dr. John Martin, now at Kearney State College, for his assistance in the early research of this project.

RJR
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INTRODUCTION

Since the time of its discovery the Dutch elm disease (DED) has plagued man by killing millions of American elm trees. This pathogen, Ceratocystis ulmi (Buisman) C. Moreau, has acquired the special attention of several botanists and pathologists. We are knowledgeable of the pathogen's life cycle as well as its vectors. Much detail has been gathered on the influence of various chemical and physical factors that affect the morphology and physiology of the DED fungus.

The causal organism of DED was first considered to be an imperfect fungus and was placed in the genus Graphium; however, as the pathogen was further investigated, the perfect (sexual) stage was found. Sexual reproduction results in ascospor production in a perithecium. The conidia, the asexual spores, are formed by abstriction at the ends of specialized hyphae called conidiophores.

McMullen (1969) studied the growth of the DED fungus on host tissue cultures in hope of deriving an efficient screening method for determining disease resistance. In his work, McMullen observed that coremia appeared only on callus tissue and in a small area on the agar surrounding the callus. He postulated that coremia formation requires factors which are supplied by certain callus cultures that are not available in the nutrient medium used.

The purpose of this study was to gather as much knowledge of any factor or combination of factors present in American elm wood that influences sporulation of *C. ulmi*. The ultimate goal of this research
is to isolate and identify a "natural substance" from American elm
wood that influences sporulation. It has been concluded by Zentmyer
(1942), Zentmyer and Horsfall (1942), Dimond (1947) and Beckman
(1956) that the DED fungus produces toxic substances. This research
was done under the following assumptions: the DED fungus metabolizes
this "natural substance" and produces a toxic by-product that acts
similar to a systemic herbicide, or that a polymeric substance is
produced that contributes to the occlusion of vessels by gums and
tyloses in accordance with Zentmyer et al. (1946), Beckman et al.
(1953), Dimond and Waggoner (1953) and Scheffer and Walker (1953).
LITERATURE REVIEW

No other North American shade tree matches the unique silhouette of the American elm, *Ulmus americana*. The American elm is widely distributed from Newfoundland down to western Texas but is unknown in Western North America. It has been called white elm, water elm and swamp elm which clearly labels its preference for the deep rich and moist soils near streams. The elm has an extensive use commercially in cooperage, flooring, shipbuilding and for certain farm implements. Stating from a landscape viewpoint Hudak (1969) said, "There is a no more unique and useful tree in North America".

It seems unbelievable that the day would come when a menacing fungus would threaten America's beloved shade tree to the point of extinction. The DED now threatens all native and European elm species in the United States.

The DED is caused by a member of the Ascomycetes and is also known as *Ceratostomella ulmi* and *Graphium ulmi*. Schwartz (1956) describes *C. ulmi* as the perfect stage of *Graphium ulmi*.

The DED was found in Holland in 1919 and it spread rapidly in Europe. According to Beattie (1933) the pathogen was carried in burl logs to North America where it was identified in Ohio by May (1930).

Efforts to control this disease include destruction of infected trees, pruning infected limbs and destroying, debarking, or spraying elm logs. But man's impetuous action to control the disease with these methods has not succeeded. Since reaching North America, the
pathogen has spread throughout most of the natural range of the American elm. It was positively identified by Randall et al. (1969) in eastern South Dakota during the summer of 1967 and is annually creeping farther west.

Rood (1969) reported that the fungus, deadly as it may be, is apparently powerless to penetrate unbroken bark of healthy trees. It must be transported to its victim and actually placed in a wound of some sort.

Two elm bark beetles, Scolytus multistriatus Marsham, the smaller European elm bark beetle, and Hylurgopinus rufipes Eichhoff, the American elm bark beetle are the principal vectors of the pathogen according to Collins (1941). Leach (1953) concluded that in the United States the principal vector, due to its feeding habits, is the smaller European elm bark beetle. The beetles, widespread in the eastern half of the United States, are present in many places where the DED is not yet known to occur - an ominous warning.

The adult beetles feed in parts of living elm trees, but they breed only in recently cut, dead or dying elms according to Collins (1941) and Rood (1969). When the DED fungus occurs in elm material in which these insects breed the fungus may stick to the beetles and be carried to healthy elms or other breeding material. Leach (1953) observed that the fungus may overwinter in the vectors body, withstanding the digestive fluids in the insects. However, tests run by Parker et al. (1940) showed that C. ulmi was obtained from the
outer surfaces of *S. multistriatus* adults in higher percentage than from their intestinal tracts.

The pathogen spreads throughout the tree by means of three types of spores described during the imperfect stage by Clinton and McCormick (1936): coremiospores; conidia which originate singly or in cephalosporium-like slimy heads on branches conidiophores and spores that bud in a yeast-like manner. They also observed "glistening bodies", which behaved like spores inside typical spores and hyphae. Ouellette and Gagnon (1960) and Wilson (1965) also observed these minute cytoplasmic particles, microendospores. Ouellette (1962a) concluded that microspores are formed exogenously and endogenously in the host tissues. Ouellette and Gagnon (1960) observed them budding or dividing which gave them the appearance of bacteria and they observed them giving rise to small short hyphae (microhyphae) when free or still enclosed in spores and hyphae.

Hart (1962) described coremia as creamy bulbous droplets of spores on top of single or branched black stalks.

The DED fungus lives as a saprophyte for a considerable part of its normal life cycle. During this phase the organism is subject to the influence of a number of environmental factors that may greatly affect its existence.

Zentmyer and Wallace (1944) reported that nutritional and anatomical differences in American elm wood and dosage and age of fungus spores are important factors influencing initiation and development of the DED. According to Westveld (1933) and Kais et al.
(1962) soil type, moisture holding capacity and water availability greatly influence both degree of infection and duration of susceptibility to DED. Zentmyer et al. (1946), Smalley (1963) and Pomerleau (1965) reported that the disease develops more readily in the spring and early summer. Brown et al. (1963) and Banfield (1968) postulated that this was due to the more rapid movement of spores in the larger spring wood vessels.

Smalley (1963) reported that increased nitrogen extended the duration of the susceptible period and greatly increased intensity of external symptoms. Singh and Smalley (1966, 1967, 1968) observed 21 amino acids and ammonia in the xylem sap of healthy and Dutch elm diseased elms. They found that proline was present in only trace amounts in the xylem sap of Ulmus species susceptible to DED, but constituted large percentages of the total amino acids and ammonia in the sap of resistant and immune species. Singh and Smalley (1969) reported accumulation of proline, alanine, and gamma-amino-n-butyric acid and reduction of amide nitrogen in the sap of elms inoculated with C. ulmi occurred more rapidly in mid-June inoculations than in late-July inoculations. Elgersma (1967) postulated that the high amino acid content of the xylem sap of resistant elms may stimulate the plant to rapid production of gums or may form a more favorable nutrient condition for the spores, resulting in a high level of toxin production inducing rapid gum formation. Thus, spore transport was limited.
Changes in the carbohydrate content of plant tissues are also affected by pathogenic agencies according to McCombs and Winstead (1964) and Singh and Smalley (1969). However, Elgersma (1967) detected no obvious differences in the sugar content of xylem saps from susceptible and resistant clones of *Ulmus hollandica*. Singh and Smalley (1969) suggest that sugar concentration changes reflect the fact that the elm tree is being subjected to extremes of water stress during pathogenesis. Ouellette (1962b) postulated differences in concentration or nature of the free sugars might explain seasonal dissimilarity in the growth habit of the fungus. The spores and hyphae were much smaller in a medium containing filter paper as the sole source of carbon than in media containing simple sugars. Feldman *et al.* (1950) observed a temporary delay in wilt symptoms when a sucrose solution was sprayed on leaves or injected into the trunk of affected elm trees. Ouellette (1962b) suggests that since brown spores appeared to be quiescent in living trees, the development of pigment in spores might restrict their invasion into xylem tissues.

Phytotoxic material present in the filtrate of a nutrient medium in which *C. ulmi* had been grown was discovered by Zentmyer (1942) and the production of toxic substances has been confirmed by Feldman *et al.* (1949) and Beckman (1956). Investigations by Zentmyer and Horsfall (1942) suggested that this thermostable toxic material consisted of more than one component. Two toxic substances obtained from culture filtrates were identified by Dimond (1949): a
polysaccharide which caused upcurling of leaves and an alcohol soluble, ether insoluble fraction which causes interveinal necrosis. Feldman et al. (1949) and Lafayette and Howard (1951), however, considered the polysaccharide as rather unimportant in relation to the pathogenicity of the fungus because the removal of the component did not reduce phytotoxicity of the filtrate. They found that the highest toxin production occurred at pH 4.25 in 7 days, and that a higher pH prevented toxin formation and deactivated preformed toxin. C. ulmi always lowered the pH of culture media, therefore the affect of pH on the pathogenicity of the fungus could not be tested.

Beckman (1956) has reported that the DED pathogen produces auxin in culture. Gothoskar et al. (1955), Waggoner and Dimond (1955), Beckman (1956) and Husian and Kelman (1957) hypothesized that exoenzymes probably are produced by wilt pathogens since they must be able to degrade organic material in order to subsist as saprophytes. These same enzymes could cause a shift in the normal host metabolism forming metabolites that induce vascular occlusion and wilt.

Physiological as well as morphological factors appear to be responsible for the severity of the host-pathogen interaction according to Banfield (1941) and Zentmyer et al. (1946).

Beckman (1958) suggests that the physicochemical condition of living cells of the host is a factor in host-pathogen interaction due to the fact that elm trees are most susceptible to infection during the period of rapid spring growth. Therapeutic agents may increase disease resistance by altering this physicochemical state. Beckman
(1958) postulated a consistent association between inhibition of sapwood development and resistance to infection.

There is considerable evidence that a primary factor in wilting is the occlusion of vessels by gums and tyloses according to Zentmyer et al. (1946), Beckman et al. (1953), Dimond and Waggoner (1953) and Scheffer and Walker (1953).

Banfield (1941) has demonstrated that spores are responsible for the rapid spread of the fungus within the tree, but they have seldom been seen in vessels. Banfield and Smith (1936) and Clinton and McCormick (1936) observed that the hyphae grew mainly along vessel walls, spreading from vessel to vessel through bordered pits. According to Banfield and Smith (1936) growth of the fungus was observed in tissues other than vessels, except when wood blocks were inoculated.

Because of their scarcity, mycelium and spores have not been considered abundant enough to plug vessels and cause wilting. Clinton and McCormick (1936) and Pope (1943) have suggested that wilting may be caused by gums, tyloses or cytoplasm from parenchyma cells which plug the vessels. Zentmyer (1942) and Zentmyer et al. (1946) postulated the production of toxins as the basic cause of wilting. On the other hand, Beckman (1956) stated that degradation of cell walls might be an important factor in the wilt mechanism since the fungus produces pectinases and cellulases. It seems likely according to Ouellette (1962b) that acute symptoms of the disease could result from the plugging of smaller vessels by spores and
mycelium of the pathogen, alone or in combination with cytoplasm and residues from adjoining cells and particles arising from the deterioration of cell walls. Ouellette (1962b) stated that extensive plugging undoubtedly could impair water movement, but all the conductive tissues would need to be almost completely plugged to cause acute wilting of distant branches. Other observations by Ouellette (1962b) suggest that physiological factors of a seasonal nature exist within the tree which may either delay infection at least temporarily, or stimulate the growth of the fungus at certain times of the year.

Xylem discoloration is one of the first symptoms of the American elm disease induced by _C. ulmi_ and apparently an important step in the development of the infection. Ouellette (1962b) observed that spores and hyphae of _C. ulmi_ may turn brown, and he reported that the brown coloration assumed by spores and hyphae is responsible for at least part of the brown discoloration observed in diseased tissues of American elms. Dark colored gums also contribute substantially to vascular discoloration, which according to Dimond (1955), may be the result of the oxidation of phenols which polymerize to form melanoid pigments. Some of these phenolic compounds could arise through the action of beta-glucosidases on glycosides.

Gagnon (1967) reported that in healthy elm trees, polyphenols are present in small amounts in newly differentiated cells near the cambium, and in higher concentrations in very few longitudinal
parenchyma and ray cells, but are generally abundant in cells of the medullary sheath. The latter occurrence is in accordance with the general view that the cells of the medullary sheath are filled with tannins, which are phenol derivatives stated by Esau (1953). In diseased trees, Gagnon (1967) observed that the high content of polyphenols in the cells that later showed pathological discoloration is evidence that this is due to oxidized phenolic compounds.

Gagnon (1967) postulated the formation of polyphenols in diseased elms does not appear to be a direct effect of the fungus since the injection of dilute alcohol or buffer solutions resulted in a similar production of polyphenols. Uritani (1963) postulated polyphenols form as a result of the increased activity of enzymes of the pentose phosphate pathway, which supplies precursor molecules for the synthesis of polyphenolic compounds. Denaturation of protein molecules may also contribute to the reaction for polyphenols by the liberation of phenolic groups of tyrosine.
METHODS AND MATERIALS

Stock cultures of a pure strain of C. ulmi were obtained from the Plant Science Department at South Dakota State University. The inoculum was maintained on Potato Dextrose Agar (PDA) in petri dishes (Fig. 1,A). On this medium, the fungus produced a grayish white rather sparse and appressed mycelium. Conidia were produced in heads on branched conidiophores. Quite often some sectoring was observed on PDA (Fig. 1,B). Cultures producing coremia, obtained on 2% American elm shavings (on dry weight basis) plus bacteriological agar (8 g/l), were also used as an inoculum source. As is shown in Fig. 2, this medium produced coremia and very little vegetative mycelium. The zonate character of the growth of the fungus (Fig. 1,C) on every concentrated elm-water extract is in response to alternate light and dark periods.

Aseptic methods were essential in this research. All inoculating was done in a White Roomette, of which the inside surfaces were washed down with 70% ethyl alcohol. Instruments were flamed with a bunsen burner periodically during inoculations. Media and petri dishes were autoclaved for 20 minutes at 15 psi. All cultures were grown under laboratory light conditions at 25°C for 14 days. Some materials and equipment used in this research are shown in Fig. 3.

Roster, Holden and Martin (1969), McMullen (1969) and Hubbes and Pomerleau (1969) hypothesized that C. ulmi produced coremia only if grown in contact with American elm tissue and that a coremia-inducing factor (CIF) was present. To test this hypothesis further,
Figure 1. A-B, C. ulmi grown on PDA. A, Notice the absence of coremia. B, sectoring. C, Concentric ring growth of fungus on concentrated elm-water extract.
Figure 2. Coremia production on 2% American elm shavings.
Figure 3. Materials and equipment used in research.

Sterilized wood disks 4 mm thick obtained from American elm twigs 2 cm in diameter, were placed in petri dishes containing PDA and the inoculum was placed directly on the disk. Wood disks of apple, plum, walnut, silver maple and green ash were also cultured in the same manner.

Wood shavings were obtained from logs of American elm (20-36 cm in diameter) with a jointer-planer. The shavings were then blended into still finer particles with a Waring blender.

Several methods were devised to test the possibility of extracting a "CIF" from American elm which contributes in some way to the production of coremia. Figure 4 shows the various solvents used in extracting.
Figure 4. Solvents used in attempting to isolate "CIF". Plus (+) indicates some coremia production. Asterisk (*) indicates maximum coremia production.
Soxhlet extraction with alcohol and with distilled water was tried. Ten grams of shavings suspended in 150 ml of solvent were refluxed for 48 hours, filtered and cultures made of both filtrate and residue.

A water extract was prepared by autoclaving 200 grams of shavings suspended in twice as much water for 12 hours at 15 psi. After filtering, cultures were made from the filtrates. The preceding procedure was repeated with the addition of 1 N HCl. The filtrate obtained in this case was a much darker red color.

In another method 200 grams of shavings were suspended in twice as much water and placed at 5°C for 72 hours. This was also done with ethanol. After filtering, cultures were again prepared from the filtrates.

Blending 50 grams of shavings in 150 ml of water for 10 minutes and filtering was eventually established as the most convenient and productive method of extraction (Fig. 4).

Flow charts of methods of extraction from the elm-water extract used throughout this research are shown in Fig. 5A-C.

In all the preceding methods, the filtrates were evaporated under vacuum in a flash evaporator with the residues being redissolved in deionized water. Quite often centrifugation at 18,000 rpm for 20 minutes was relied upon to eliminate residue or precipitate. This procedure was also used in future extraction methods.
Figure 5, A. Flow chart of dialysis extraction.
Figure 5,B. Flow chart of ion exchange chromatography and lead acetate extraction.
Figure 5C. Flow chart of charcoal and butanol-acetic acid extractions.
Since the elm-water extract was shown to contain the "CIF", the research was directed toward isolation of this factor and an attempt was made to determine whether it is a substance or a combination of chemical and physical factors.

Forty ml of extract (100 g/l) was mixed with 2 grams of animal charcoal, filtered and cultures made of both filtrate and residue. Since sporulation was observed on the charcoal, attempts were made to elute the "CIF" off the charcoal. The charcoal residue was suspended in water and adjusted to pH 8.0 with barium hydroxide, the suspension was filtered and the flask containing the filtrate was marked A. The remaining charcoal residue was resuspended in water, the pH was adjusted to 10.0 with barium hydroxide, the suspension was filtered and the flask containing the filtrate was marked B. The charcoal residue was again resuspended in water, the pH was adjusted to 12.0, the suspension was filtered and the filtrate was marked C. The remaining charcoal residue was marked D. The pH of all 3 filtrates and the residue were adjusted to pH 7.0 with sulfuric acid and then cultured. This same procedure was repeated on the acidic side using sulfuric acid to adjust the pH of the filtrates to 5.0, 3.0 and 1.0. These filtrates along with the remaining charcoal residue were adjusted to pH 7.0 with barium hydroxide. Ethanol solutions (50% and 100%) were also used in attempting to elute "CIF" from the charcoal.

Ion exchange chromatography was used to test the possibility of the presence of acidic and/or basic substances in the elm-water extract which contribute to the production of coremia. Amberlites
IR-45 and IRC-50 were activated with 1 N NaOH and L N HCl respectively, placed in columns and backflushed with distilled water. After the extract had passed through the columns, they were washed with distilled water, leaving behind only those compounds ionically bound to the ambers. The adsorbed substances were removed by regenerating IR-45 and IRC-50 with NH₄OH and HCl respectively. All fractions obtained were flash evaporated to dryness and residue was redissolved in water.

The possible role of flavonoids and related compounds was investigated using 50 ml of elm-water extract (100 g/l) plus enough lead acetate to make a 0.1 N solution. The precipitate formed (flavonoids) was suspended in water. Hydrogen sulfide was bubbled throughout the mixture causing the formation of a black precipitate (lead sulfide). Cultures were made of the resulting supernatant which contained the flavonoids. The supernatant of the original extract obtained from precipitating with lead acetate was cultured as the control. Hide powder and gelatin plus 10% NaCl were also used to remove tannins in both column and bulk procedures. Flavonoids were separated from the tannins with ethyl acetate.

An attempt was made to gain some knowledge of the molecular size of the active substance by dialyzing the elm-water extract. The cellophane sack containing the extract was immersed in a large volume of water which was changed 4 times in 48 hours. Cultures were made of the colloidal substances remaining inside the tubing and of the
crystallloidal particles that diffused through the differentially permeable membrane. The latter was concentrated before being cultured.

Both column and paper chromatography methods were used to isolate this "CIF" from the elm-water extract.

In column chromatography, the stationary phase consisted of wet starch or wet silica gel, and the moving organic solvent was butanol-acetic acid (4:1). The stationary phase was removed from the column and placed under ultraviolet light where the fluorescent area was removed and eluted off the starch or gel with water-butanol-acetic acid (5:4:1) and filtered. The filtrate was evaporated under vacuum, the residue redissolved in distilled water, adjusted to pH 7 and cultured.

Whatman paper #3 was used in the chromatography experiment.

The compilation given below includes solvents which were applied.

(All proportions are given by volume.)

1. n-Butanol-acetic acid-water (4/1/5).
2. n-Butanol-pyridine-water (10/3/3).
3. n-Butanol saturated with 2% aqueous ammonia.
4. Forestal solvent. Conc. hydrochloric acid-acetic acid-water (3/30/10).
5. Ethyl acetate-pyridine-water (12/5/4).
6. Benzene-acetic acid-water (2/2/1).
7. Toluene-acetic acid-water (4/1/5).

Below is a list of spraying reagents used to obtain color reactions. Ultra-violet light was also relied upon as an identifying source.

1. Methyl red
2. Ferric chloride
3. Ammonia vapors
4. Diazotized sulfanilic acid
5. Sodium molybdate
6. Bisdiazotized benzidine
7. Universal indicator

Several known compounds were spotted beside the unknown for identification purposes.

In still another method, 250 ml elm-water extract from 50 grams of shavings, was poured in a separatory funnel containing butanol-acetic acid (4:1 v/v). The mixture was swirled and stored at 5°C until a lower water soluble layer and an upper butanol-acetic acid layer had been separated by an emulsion layer. Each layer was evaporated under vacuum, the residue redissolved in distilled water, adjusted to pH 7 and cultured.

Additional experiments were conducted to determine the effect of various phenolic compounds and their derivatives at 1 g/l on the production of coremia. Below is a list of compounds used:

1. tannic acid
2. quercitrin
3. gallic acid
4. shikmic acid
5. catchin
6. quercetin
7. pyrogallol
8. phloroglucinol
9. resorcinol
10. vanillic acid
11. catechol
12. coumarin
13. chlorogenic acid
14. caffeic acid
15. esculin
16. ferullic acid
17. hydroquinone
18. cinnamic acid

These compounds were also added individually to the following medium (modified from Zentmyer, 1942) which was diluted to 1 liter with half-strength Hoagland solution.

10 g. glucose
2 g. asparagine
5 g. malt extract
To study the influence of light on coremia production, cultures containing 50 g/l and 100 g/l elm-water extract (by dry weight) were subjected to various lengths of light exposure after growing 7 days in total darkness at 25°C. After light treatment the cultures were again placed in total darkness for another 7 days.

Cultures containing 100 g/l of elm-water extract were grown in complete darkness for 16 days. After 7 days some of the cultures were subjected to 5°C for 48 hours while others were subjected to 36°C for 48 hours.
RESULTS

This writer has limited his study to the imperfect stage of *C. ulmi* because the imperfect asexual stages carry the burden of propagating and disseminating the species. Throughout the spring and summer, several generations of conidia are being produced. Often the imperfect stage is represented by numerous coremia bearing thousands of conidia (coremiospores) in droplets (Fig. 6, A). In this instance a group of conidiophores unite at the base and part way up the tip (Fig. 6, B) and form a dark-stalked structure called a synnema. The top of the synnema is branched with colorless, flaring heads, to which conidia adhere as white to yellowish droplets. The stalk of the fructification is longer in comparison to the branched top, and the fruiting body resembles a black, long-handled feather-duster (Fig. 6, C). The factors influencing the production of coremia by *C. ulmi* was the concern of this thesis.

Roster, Holden and Martin (1969), McMullen (1969) and Hubbes and Pomerleau (1969) postulated that the production of coremia by *C. ulmi* was directly or indirectly related to the presence of extracts of American elm. It was therefore hypothesized that a "coremia-inducing factor" is present in American elm wood. An attempt was made to develop an efficient method of extracting the "CIF" from the wood. In accordance with McMullen's (1969) postulation, that the "CIF" is soluble in polar solvents, several methods of extraction (Table 1)
Figure 6. Asexual fruiting bodies (coremia). A, Bulbous droplets containing conidia on terminal ends of synnemata, top view, X40. B, Synnema, note the mat of mycelium at its base, X100. C, Conidia (coremiospores) form along tips of branched hyphae at distal and of a synnema, X430.
Table 1. Extraction procedures and the relative amount of coremia formed after growth on agar medium.

<table>
<thead>
<tr>
<th>METHOD</th>
<th>cold water extraction</th>
<th>autoclaved water extraction</th>
<th>cold alcohol extraction</th>
<th>Soxhlet extraction with water</th>
<th>Soxhlet extraction with alcohol</th>
<th>extraction by blending with alcohol</th>
<th>extraction by blending with water</th>
</tr>
</thead>
<tbody>
<tr>
<td>COLOR</td>
<td>red-orange</td>
<td>red-orange</td>
<td>light yellow</td>
<td>red-orange</td>
<td>bright yellow</td>
<td>bright yellow</td>
<td>red-orange</td>
</tr>
<tr>
<td>COREMIA</td>
<td>++</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
</tr>
</tbody>
</table>

*+ indicates the degree of total coremia production*
were tried using polar solvents. The different colors of the extractions are also included in this table.

It was observed that the coremia did not grow evenly throughout the medium and quite often they were found only at the periphery of the petri dish.

Extraction in a Soxhlet apparatus showed maximum coremia production (Table 1). Because of the amount of time required for this procedure, the water extract obtained from blending was the method of extraction used throughout most of this research (Fig. 4).

All species of elm native in the United States are susceptible to the DED fungus. It would seem that the "CIF" may not be present in other woods. To test this hypothesis the fungus was grown on wood disks of various angiosperms. Table 2 shows that all species tested produced coremia.

Coremia were present on the disks but none were found on the PDA (Fig. 7). This experiment shows that a relationship exists between coremia formation and American elm wood. Also, Table 2 offers strong evidence to the fact that other woods have the ability to provide the "CIF".

Outerbark, sapwood and heartwood shavings from American elm were each cultured and observed for coremia. All three proved to be excellent substrates for coremia production.

Serial dilutions were made to determine what concentrations of American elm extract are necessary for coremia production (Table 3). Scarce coremia production was observed at concentrations as low as
Table 2. DED fungus grown on wood disks of angiosperms. Disks of wood (2 cm. in diameter) were placed in petri dishes containing PDA and the inoculum placed directly on the disks.

<table>
<thead>
<tr>
<th>Chinese Elm</th>
<th>Slippery Elm</th>
<th>Apple</th>
<th>Plum</th>
<th>Black Walnut</th>
<th>Silver Maple</th>
<th>Green Ash</th>
<th>PDA</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

+ indicates coremia were present
- indicates no coremia present

Figure 7. Coremia formation on American elm wood disks, X20.
5 g/l. The coremia were immature at this concentration, i.e., the conidia were borne on very short light-colored synnemata. An interesting observation in cultures containing 1 g/l was the presence of dark mats of mycelia embedded within the medium (Fig. 10, A) but did not protrude above the surface of medium as synnemata.

In regard to the results obtained in Table 3, a synthetic medium was used with the various concentrations of elm-water extract. The purpose was to see if the presence of a synthetic medium would lower the concentration of extract required for the production of coremia. There was a definite increase in somatic growth and possibly in the production of conidia on conidiophores, but a change in the number of coremia present was not apparent.

Table 3. DED fungus grown on serial dilutions of elm-water extract in an agar medium.

<table>
<thead>
<tr>
<th></th>
<th>50 g/l</th>
<th>10 g/l</th>
<th>5 g/l</th>
<th>1 g/l</th>
<th>0.5 g/l</th>
<th>0.1 g/l</th>
</tr>
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<td>++ +</td>
<td>+</td>
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</tbody>
</table>

+ indicates the degree of total coremia
- indicates no coremia

Animal charcoal (2 grams) was added to 50 ml of elm-water extract in an attempt to further isolate the "CIF". Charcoal was used because of its high affinity properties. Cultures made from the filtrate and charcoal residue in the presence of nutrient agar showed the absence of coremia on the filtrate and the presence of coremia on
the charcoal residue. Attempts to release the "CIF" from the charcoal were unsuccessful. The pH of the charcoal residue was adjusted to 8.0, 10.0 and 12.0 with barium hydroxide and filtered each time. The same procedure was carried out on the acidic side at pH 5.0, 3.0, and 1.0 with sulfuric acid. The filtrates and final residue were cultured on nutrient agar. Results showed coremia only on the residue. The same results were obtained when ethanol was used to elute the "CIF" from the charcoal.

Dialyzation of the elm-water extract proved to be an excellent procedure for eliminating several substances. After 14 days, coremia appeared in abundance on cultures made of dialyzed extract while few coremia were observed on undialyzed extract (Fig. 8,A). It is quite possible that several more rinsings might have eliminated all signs of coremia in the latter.

Addition of lead acetate to elm-water extract produced a precipitate that, when cultured, showed a luxuriant growth of coremia. Better results were obtained if the extract was dialyzed first (Fig. 8,B). Lead acetate is known to precipitate phosphates and phenols but phosphate analysis of the dry sawdust and the isolate obtained by precipitating with lead acetate yielded these results: sawdust = .012% phosphate; lead isolate = .007% phosphate. It would therefore seem quite improbable that this very small amount of phosphate could have much influence on the DED fungus producing coremia. The precipitate gave a positive phenolic test with Millon's reagent.
Figure 8. A, Effect of dialyzing elm-water extract on the production of coremia: left, portion of extract that diffused through differentially permeable membrane, light area is actually numerous coremia; right, portion of extract that did not diffuse through differentially permeable membrane, shows very few coremia. B, Effect of lead acetate on the production of coremia when added to the elm-water extract that diffused through differentially permeable membrane: top, diffused through membrane; lower left, portion of extract that did not form a precipitate with lead acetate, no coremia present; lower right, portion of extract that formed a precipitate with lead acetate, abundant coremia with noticeably darkened hyphae.
To further test the hypothesis that the "CIF" might be a tannin or tannin derivative, the elm-water extract that diffused through the differentially permeable membrane was precipitated with gelatin and sodium chloride. Coremia were observed on cultures made of both supernatant and precipitate; however, only a few were observed on the latter.

Ethyl acetate was added to the supernatant obtained from above with the resulting ethyl acetate soluble portion showing excellent coremia production while no coremia were observed on the aqueous remainder.

In another test, HCl was added to the supernatant obtained from adding gelatin-sodium chloride to the extract that diffused through dialysis tubing. Autoclaving this supernatant for one hour resulted in the formation of a red-brown precipitate. Then ethyl acetate was added to the supernatant with the same results occurring as above.

An elm extract added to a column of hide powder resulted in abundant coremia production on the effluent, some on the eluate and none on the hide powder which served as the control.

Ion exchange chromatography, both column and batch techniques, using amberlite IRC-50 (cation exchanger) and IR-45 (anion exchanger) as resins served as another tool for separating the elm-water extract. Coremia were observed on cultures made of both effluent and the eluate obtained from regeneration of IR-45 while only the effluent of IRC-50 showed sporulation. These experiments were repeated several times,
often resulting in contradictory results; however, coremia were never witnessed on cultures of eluate obtained from regeneration of IRC-50.

Paper chromatography was another separation technique used in this study. The following "CIF" sources were used to spot chromatograph paper: (1) American elm-water extract, (2) dialysis extract, (3) lead acetate precipitate, (4) portion of extract not soluble in butanol-acetic acid and (5) chloroform extract. It was realized that the composition of the solvent used for optimum separation of components depended on the nature of these components and the complexity of the mixture. Therefore, solvents of many compositions were applied in this study but separations obtained were not at all clear and color forming tests did not give conclusive results. Several phenolic compounds were tested with the unknowns. In the lead acetate precipitate test, a blue fluorescent spot was observed having an Rf value close to that of caffeic acid. The "CIF" seemed to have fluorescent abilities when in extract form, but when chromatographed the fluorescence did not show up as distinctive spots, but rather as a horizontal line across the solvent front.

Thin-layer chromatography was also tried with the same results as above except that the chloroform extract gave distinctive spots under ultra-violet light.

Further separation was attempted with columns of silica gel and cellulose through which elm-water extracts were passed. These procedures were repeated several times and cultures made of both effluent and eluate. Coremia were always observed on cultures made
of effluents and sometimes on cultures made of eluates. However, abundant coremia were apparent twice on cultures made of eluate obtained from silica gel. In the latter case a brown ring was apparent which fluoresced under ultra-violet light.

Butanol and acetic acid added to an elm-water extract resulted in a solution containing 3 partitions (Fig. 9,A). When cultured the water portion had abundant coremia (Fig. 9,C and D) while the emulsion had a few coremia and the butanol-acetic acid portion (Fig. 9,B) had none. When the experiment was repeated under constant light, the butanol-acetic acid portion produced some coremia. The color of the concentrated water extract changed from a dark brown to a dark red-orange while the butanol-acetic acid changed from colorless to a light red. Also, the growth of mycelium on a control of elm-water extract appeared white while the elm-water extract cultured after separation with butanol-acetic acid had black mycelium and many more coremia. Larger coremia were more apparent on the darker portions of the mycelial mass (Fig. 9,C-D).

Unsuccessful attempts were made to devise a synthetic medium that would stimulate coremia production.

The DED fungus was cultured on each of the various phenolic compounds and phenolic derivatives (see Methods and Materials) with and without the addition of synthetic media. Negative results were observed on all compounds except tannic acid. Throughout this research, the presence of brownish-black mycelial mats (Fig. 10,A-B) was observed just beneath the surface of
Figure 9. Liquid-liquid separation of elm-water extract with butanol-acetic acid (4:1 v/v). A, Separatory funnel containing: top, butanol-acetic acid layer; middle, emulsion layer; bottom, water layer. B, Culture of butanol-acetic acid layer, notice no sign of spore production and very sparse mycelial growth. C-D, Cultures of water layer. Notice the extra large coremia on the darker portions of the mycelium.
Figure 10. Coremia development. A, Dark mat of mycelium, X100. B, Dark mats of mycelium, X50. C, Coremia in its entirety, X100.
the medium. These dark colored mats usually appeared at the base of synnemata; however, they were occasionally observed in cultures that did not produce coremia. It was hypothesized that the "CIF" was involved in forming these mats of hyphae which will form thick sporophores (Fig. 10,C).

Light was observed to be a limiting factor in coremia production. Elm-extract cultures grown in constant light (Fig. 11,A) showed coremia throughout the medium while those grown in total darkness (Fig. 11,B) produced no coremia, although somatic growth was much faster in the dark. Some cultures were grown in the dark for 7 days and then exposed to light for different lengths of time and then placed back in the dark for the duration of the experiment. Light exposures as short as 10 minutes (Fig. 11,C) produced a ring of coremia.

Elm-extract cultures were grown for 16 days in complete darkness but after 7 days they were subjected to a temperature of 5°C for 48 hours. The ring of coremia observed (Fig. 11,D) was very similar to that produced by short light exposures. Cultures subjected to 36°C did not have coremia present.

Changes in carbohydrate content seems to have little effect on this pathogenic fungus. At high concentrations of sucrose (30 g/l) the coremia appeared to have abnormally large bulbous droplets but no difference in number of coremia present from that of control was observed.
Figure 11. A-C, Effect of light on coremia production on elm-extract cultures. A, Culture grown in constant light; B, Culture grown in total darkness; C, Culture grown in total darkness with 10 minutes of light exposure after 7 days. D, Culture grown in total darkness at 25°C with 48 hours of exposure to 5°C temperature after 7 days. All cultures were observed after 14 days.
Addition of enzymatic casein hydrolysate resulted in restriction of coremia as well as inhibition of fungal growth at concentrations greater than 0.5 g/l.

An amino acid analysis of American elm extracts on an amino acid analyzer revealed the presence of varying small amount of several amino acids along with noticeably larger amounts of proline. Cultures made from these extracts showed abundant coremia production.

Serial dilutions of alpha-naphthalene acetic acid (NAA) added to 2% elm shavings yielded the following results. Concentrations of 10 mg/l, 50 mg/l and 100 mg/l appeared to increase coremia production. However, actual counts indicated that the number of coremia had not increased noticeably from that of the control (Fig. 2), but the size of those present were much larger (Fig. 12,A). The mycelial mass darkened considerably at higher concentrations and fungal growth was inhibited at 0.5 g/l and above. Alpha-naphthylamine hydrochloride (100 mg/l) and beta-napthoxy acetic acid (100 mg/l) yielded the same results as NAA. Alpha-naphthyl acetamide (Fig. 12,B) not only darkened the mycelium but also blackened the entire substrate. Alpha-naphthol (100 mg/l) inhibited fungal growth. NAA added to PDA cultures showed coremia formation at a concentration of 50 mg/l (Fig. 12,C).

Carbanilide (s-diphenylurea) added to 1% elm shavings (Fig. 13, A-D) gave similar results to those obtained with NAA. Concentrations higher than 50 mg/l caused total inhibition, but 100 mg/l in PDA cultures produced coremia (Fig. 14,A).
Figure 12. Effects of naphthyl compounds on coremia production. A, 10 mg/l NAA plus 2% elm shavings. B, 100 mg/l alpha-naphthyl acetamide plus 2% elm shavings. C, 50 mg/l NAA in PDA medium.
Figure 13. Effect of carbanilide (DPU) on coremia production. A, Control, 1% elm shavings. B, 1 mg/l, the mycelium is beginning to darken in periphery. C, 10 mg/l, darkened mycelium is throughout the culture. D, 50 mg/l, mycelial growth is showing signs of inhibition although coremia are still very apparent.
Figure 14. A, 100 mg/l carbanilide plus PDA. B, 1 g/l ammonium chloride plus 2% American elm shavings.
Addition of ammonium chloride to 2% elm shavings (Fig. 14, B) causes a shift in habit resulting in vegetative mycelium only. Higher concentrations of ammonium nitrate produced the same effects.

Tannic acid (5 g/l) was added to a synthetic medium consisting of: 1 g/l yeast extract, 10 g/l sugar and 0.1 g/l ammonium nitrate. This synthetic medium was altered from that given in Methods and Materials. Observation of resultant cultures (Fig. 15, A) showed excessive coremia production. The coremia (Fig. 15, B) appeared to have shorter and lighter colored synnemata than those observed on elm cultures (Fig. 6 and 10). However, the addition of 10 mg/l NAA blackened the synnemata so that colorwise they were identical to those formed on elm. Serial dilutions of tannic acid, in addition to synthetic medium, showed coremia production on cultures containing a minimum of 1 g/l. Cultures containing concentrations of tannic acid higher than 5 g/l were not observed because of the difficulty in getting the medium to solidify. Light did not appear to have any effect on coremia production. Tannic acid (2.5 g/l) cultures void of synthetic medium provided an adequate substrate for neither somatic growth nor spore formation.
Figure 15. Coremia formation on a synthetic medium containing 5 g/l tannic acid. A, Dense mycelial mass with abundant coremia. B, Coremia, notice the light colored synnemata, X100.
DISCUSSION

The study includes several methods devised in an attempt to isolate the "CIF" from American elm wood. Conditions necessary to produce coremia as well as attempts to produce coremia on synthetic media were discussed. The appearance of the pigment blackening the aggregates or mats of mycelium that form synnemata was also investigated.

As indicated in Fig. 4, the "CIF" appears to be highly soluble in polar solvents. This is in agreement with McMullen (1969); however, Hubbes and Pomerleau (1969) observed maximum coremia production on chloroform extracts. Their method was tried and coremia were obtained in culture but not to the extent obtained on polar solvents. The difference could lie in the technique of extracting.

The occurrence of coremia only in the presence of light (Fig. 11, A) is verified by Hubbes and Pomerleau (1969). Constant light is not essential (Fig. 11, C) as cultures exposed to 10 minutes of light showed a ring of coremia at the periphery of somatic growth. Mycelia formed prior to light treatment never developed coremia even after the culture was exposed to light. Similar results obtained with cold treatment (Fig. 11, D) indicated the possibility of a stress factor being involved. Results indicated that "CIF" was a combination of both physical and chemical factors and that either will not produce coremia. Banfield (1941) and Zentmyer et al. (1946) also reported physiological as well as morphological factors appear to be
involved in the host-pathogen interaction. Environmental factors might initiate a shift in the normal pathogen metabolism causing it to metabolize the "CIF" obtained from the host.

Results of serial dilutions shown in Table 3 indicate that coremia can be produced on relatively low concentrations of elm-water extract. The coremia on the $5 \text{ g/l}$ cultures were identical in appearance to those on the $50 \text{ g/l}$ cultures. The difference observed was in the quantity of coremia present. Due to the observance of the black pigmented mats of mycelium embedded in the extract of $1 \text{ g/l}$ cultures, a synthetic medium was added because the added nutrients might enable these small mats to continue growth and differentiation into actual synnemata or coremia. However, this was not the case. It was hypothesized that $5 \text{ g/l}$ of elm-water extract was essential to supply an adequate amount of "CIF" for the formation of coremia.

The fact that the "CIF" appeared in the dialysate (Fig. 8, A) obtained from dialyzation indicates the molecular structure of the molecules of concern are relatively small in size.

The results observed from charcoal extraction indicated the "CIF" was tenaciously adsorbed to the charcoal. This conclusion was reached after observing coremia on the charcoal residues which tended to support the hypothesis that charcoal did not inhibit the production of coremia.

Since phenolic substances might be involved, an attempt was made to test elm-water extracts for flavonoids and related compounds, since many compounds of this group are water soluble and can be
precipitated with lead acetate. The results with lead acetate (Fig. 8,B) showed that the "CIF" was in the precipitate. Flavonoids were freed from the lead precipitate by adding hydrogen sulfide leaving the lead as insoluble lead sulfide (Robinson, 1967). He also recommended this procedure to distinguish between the hydrolyzable tannins that form a precipitate and the condensed tannins which remain in solution.

It is not uncommon for flavonoids to polymerize forming polymers involving unknown numbers of units that constitute the group of compounds known as "condensed tannins", "phlobatannins", "flavolans", or "catechol tannins". Hydrolyzable tannins are also somewhat soluble in water. However, they usually form colloidal rather than true solutions according to Robinson (1967).

In plant extracts which have already been postulated to contain phenols, Bonner and Varner (1965) stated that the presence of tannins is best shown by testing their protein binding capacity. This is done by adding a few drops of a 1% solution of gelatin containing 10% sodium chloride to a portion of the extract; a precipitate or definite cloudiness indicates the presence of a tannin. In this study a cloudiness in the elm-water extract and a slight precipitate was observed. This test is not specific, and some simple phenols do give positive reactions if present in a sufficiently high concentration. According to Bonner and Varner (1965), the absence of a precipitate is not necessarily due to lack of tannins in the extract, but might be because the pH, ionic strength and concentration are unfavorable.
Davies et al. (1964) stated that tannins should adsorb strongly to hide powder. In this study, hide powder did not support the inference that "CIF" was a tannin. However, the fault may lie in the technique and more work is presently being done on perfecting this procedure. Davies et al. (1964) also concluded that on treatment with hot acid, tannins should give a red precipitate. The red-brown precipitate observed when 1 N HCl was added to the supernatant obtained from precipitating extract with gelatin, was probably polymers known as phlobaphenes or tannin红色s.

Robinson (1967) observed re-extraction of an aqueous solution with an immiscible, rather polar organic solvent is frequently of value in separating flavonoids from more polar compounds such as carbohydrates. Upon addition of ethyl acetate to the supernatant obtained from precipitating with gelatin, coremia were produced on cultures made of ethyl acetate solution and not on the aqueous remainder. This would seem to enhance the possibility of the "CIF" being a flavonoid or related phenolic compound.

Results obtained with ion exchange chromatography were inconclusive. The fact that some coremia were produced on the eluate from IR-45 and not from IRC-50 does raise an interesting possibility. The ion exchange resin IR-45 is a weak anion exchanger. Although it may be used as an adsorbent for acidic materials of all types, weakly ionized acids such as phenols probably do not adsorb as efficiently as they would on a strong anion exchanger. More work is being done in this area.
Ammonium chloride and ammonium nitrate added to cultures of 2% elm shavings cause the DED fungus to revert to production of extensive vegetative mycelia instead of coremia. One can speculate that these compounds increase the amount of available nitrogen for protein synthesis resulting in more abundant somatic growth. It has been the observation of the writer throughout this research that a highly nutritive medium does indeed increase somatic growth of C. ulmi.

The production of coremia on a synthetic medium containing tannic acid (Fig. 15, A) proved to be very interesting since coremia are not known to appear on media void of American elm shavings or extract. The hydrolyzable tannins are often complex mixtures containing several different phenolic acids esterified to different positions of the sugar molecule. The "tannic acid" of commerce is actually a mixture of free gallic acid, gallic acid polymers and various galloyl esters of glucose (Robinson, 1967). In this study, tannic acid merck was used, obtained from Merck and Company, Incorporated, Rahway, New Jersey. Coremia formation on tannic acid (1 g/l) cultures was not consistent, although higher concentrations (3-5 g/l) always showed some coremia. Gallic acid at these concentrations showed no signs of coremia formation. The fact that NAA darkened the synnemata of coremia formed on tannic acid cultures would make one think that a polyphenol oxidase system might be involved.

It is possible that the pigmentation of the hyphae that form synnemata is due to oxidized phenolic compounds that may originate by synthesis or by degradation of existing compounds. The substrate
(elm) may contain a substance that stimulates the production of a polyphenol oxidase in the fungus. This enzyme may act on a phenolic substance already present or stimulate the production of a phenolic substance. The resulting oxidized phenolic substances then contribute to the blackening of synnemata. Another mode of action may be the hydrolysis of a phenolic glycoside. The resultant freed phenolic compound may be toxic or in turn trigger the formation of a polyphenol oxidase within the fungus which might result in the production of metabolites that affect the elm.

The absence of black synnemata when coremia are formed on tannic acid cultures lacking NAA (Fig. 15,B) would seem to indicate that oxidation of phenolic compounds has a separate role from that of coremia formation.
CONCLUSIONS

This paper includes a catalogue of events attempted during the identification of the "coremia inducing factor" found in American elm-water extracts. Although several procedures used proved to be of limited value, they were all included in order to present a step-by-step sequence of events leading up to the present level of understanding. It is hoped that this listing might be of some value in further research.

On the basis of the data presented in this investigation, the following conclusions were drawn in regard to the necessity of American elm in the production of coremia by *Ceratocystis ulmi*.

1. The "coremia inducing factor" is soluble in polar solvents and can be removed from American elm shavings with water.

2. A relationship between coremia formation by *C. ulmi* and the presence of American elm shavings or extract does exist.

3. Other angiosperms also provide the "coremia inducing factor" necessary for coremia formation. Susceptibility of the Dutch elm disease fungus and lack of a vector to spread the disease are two possible reasons for immunity of other trees.

4. Although the growth of *C. ulmi* is primarily restricted to the sapwood in an infected elm tree, coremia are formed on outer-bark, sapwood and heartwood under laboratory conditions.

5. Coremia are produced in cultures containing a minimum of 5 g/l American elm-water extract.
6. Light is a limiting factor in coremia production although constant light is not essential.

7. Exposure of cultures to 5°C temperatures for short periods of time will replace the need for light in the production of coremia.

8. The "coremia inducing factor" can be precipitated with lead acetate.

9. The "coremia inducing factor" appears to be very soluble in ethyl acetate.

10. Higher concentrations of ammonium compounds (concentrations above 0.1 g/l) tend to cause a shift in the Dutch elm disease metabolism resulting in increased somatic growth in preference to coremia formation.

11. Although several fluorescent and some phenolic compound spots have been observed, further attempts should be made to identify the unknown by thin-layer and paper chromatographic procedures.

In screening for compounds that might have "coremia inducing factor" potential, substances that are usually associated with wood were selected. The addition of these substances to the basal medium resulted in the following conclusions.

12. Tannic acid at a concentration of 1 g/l or greater tends to produce whitish-brown coremia.
13. Naphthalene analogs, such as alpha-naphthalene acetic acid tend to cause a blackening of synnemata and somatic fungal growth if higher concentrations are added. Ethyl alcohol fumes also tend to blacken coremia stalks.

14. Coremia will form on PDA with the addition of 50 mg/l alpha-naphthalene acetic acid or 100 mg/l of carbanilide.

15. Both tannic acid, identified by chromatography and naphthalene compounds (Chen and Hostettler, 1969) have been identified in elm species.

16. The production of coremia appears to result from the interaction of two factors: (a) coremia inducing substance, (b) pigment inducing substance. These two factors can occur independently but usually occur together in coremia formation. Both of these factors may be influenced a great deal by physical conditions.

In summary, a theory is proposed that conidia, cephalospires and coremiospores are related in the following manner. On a synthetic medium lacking tannic acid or elm-water extract, white conidiophores and cephalophores are formed in abundance. With the addition of tannic acid, conidiophores tend to become sticky and are cemented together forming synnemata. The addition of a physical or chemical factor (the latter present in elm-water extracts) causes the induction of a polyphenol oxidase resulting in blackened synnemata. If the resulting stress is low only the synnema and the mat of mycelium at its base
become darkened. If the stress is great enough the entire mycelial mass in the petri dish blackens. Therefore, in order for blackened coremia to be produced in elm tissue a tannic acid-like substance must be present together with a substance acting like alpha-naphthalene acetic acid. Further research is needed to determine the exact chemical nature of these compounds in American elm.
LITERATURE CITED


