Electron microscopy of elm infected with Ceratocystis ulmi (Buism) C Moreau

William Lloyd MacDonald

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ELECTRON MICROSCOPY OF ELM INFECTED WITH

*CERATOCYSTIS ULMII* (BUISM.) C. MOREAU

by

William Lloyd MacDonald

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of
The Requirements for the Degree of
DOCTOR OF PHILOSOPHY

Major Subject: Plant Pathology

Approved:

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1970
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INTRODUCTION

Dutch elm disease, caused by Ceratocystis ulmi (Buisn.) C. Moreau, has received the attention of researchers for nearly fifty years. The heavy damage it has inflicted on the elms of both Europe and North America is reflected in the voluminous literature that exists on the disease.

Generally, the literature reflects a concern for practical controls and has dealt also with the reactions of the susceptible hosts. Furthermore, studies of the internal host reaction have been conducted after expression of external symptoms. Relatively little attention has been paid to those trees that survive and even less attention to the host responses of resistant species. The study of resistance mechanisms appears to be the most meaningful direction for research to take if substantial gains are to be made in understanding the disease and, in turn, provide more practical methods of control. With this work I have intended to correlate earlier studies that have dealt with both resistant and susceptible species and to add to this knowledge of Dutch elm disease.

I have made a comparative investigation of the initial stages of infection within three elm species that vary in their susceptibility to disease. New histological techniques were employed that have aided in the interpretation of host responses. The electron microscope has provided, for the first time, the resolution necessary to substantiate previous findings and establish new ones. This study was undertaken to meet the following objectives:

1. to examine the anatomical host responses to the pathogen and
in turn relate them to observations made with the electron microscope

2. to determine any variations among elm species in their initial reaction to *C. ulmi*

3. to correlate any of these differences with the disease susceptibilities found in these elm species.
REVIEW OF LITERATURE

General Aspects

There is a vast body of literature on host responses in vascular diseases. Several general reviews are Gäumann (1951), Dimond (1955), Sadasivan (1961), Beckman (1964) and Dimond (1967). Few investigations have been concerned with Dutch elm disease and the amount of information that is applicable remains to be established. Nevertheless, concepts can be formulated and will be integrated into the Dutch elm disease literature where appropriate.

Fungal movement

The infection of elm by *Ceratocystis ulmi* (Buism.) C. Moreau initiates a series of internal host responses that proceed in the vicinity of the conidia and mycelium. The spread of responses is therefore dependent upon the movement of the fungus within the vascular tissue.

The movement of *C. ulmi* spores within the xylem vessels of *Ulmus americana* L. has been demonstrated, but the rate of movement is controversial. Rapid acropetal movement of spores (from 32 to 64 feet, 1 hour after inoculation) through the large earlywood vessels was reported when trees were bole-inoculated with a massive number of spores (Banfield, 1941). Other researchers have suggested that the acropetal movement of spores demonstrated by Banfield (1941) represented a highly artificial condition (Brown, et al., 1963; Campana, 1967). Under natural bark-beetle infection, spore movements probably occur at a slower rate. Furthermore, the beetle carrier, *Scolytus multistriatus* Marsh, while
feeding in twig crotches, could inoculate with only a limited number of spores. Records of artificial twig-crotch inoculations documented these suggestions (Brown, et al., 1963; Campana, 1967). Kerling's (1955) observations on European elm likewise have indicated that acropetal spore movement occurred at a slower rate. Basipetal movement has also been shown (May, 1935; Banfield, 1938; Hart, 1960; Brown et al., 1963; Campana, 1967; Neely, 1968). Furthermore, workers have agreed that basipetal movement is much slower than acropetal movement. In addition to the spore movements mentioned above, $^{32}$P-labeled spores have been observed to move into petioles and leaves of *U. americana* (Pomerleau and Mehran, 1966). Undoubtedly spore movements within the xylem vessels have appeared erratic because the transpiration stream is erratic (Buisman, 1936; Banfield, 1938; Hart, 1960).

Vascular invasion by *C. ulmi* is influenced both by the time and locus of infection. The dissolution of the end walls of earlywood vessels has been correlated closely with the initiation of the period of susceptibility of *U. americana* (Banfield, 1938; Campana and Hyland, 1970). Early cambial activity was related to the susceptibility period of elms in Quebec, Canada (Pomerleau, 1965, 1966). In contrast, the increase in resistance during the growing season appeared closely related to the onset of late-wood production (Pope, 1943; Banfield, 1968).

The locus of infection likewise was shown to be a factor in distribution of *C. ulmi* spores (Banfield, 1947). Vascular invasion was limited when trees 1.5 to 4.0 in. in diameter at breast height were inoculated in the crowns; however, extensive invasion occurred where inoculations were made in the boles or roots. Histological studies revealed that the extent
of vascular invasion was limited by vessel length at the point of inoculation (Banfield, 1941). Vessels in new shoots were only a few centimeters long in contrast to those in the bole which reached lengths of several meters (Priestley et al., 1935; Banfield, 1941; Greenidge, 1952).

Lateral movement of *C. ulmi* must be involved in diseased trees if complete colonization is to occur, but such information is limited. Only a few investigators have reported spores or mycelia within the vessels of naturally infected elm (Schwarz, 1922; Wollenweber, 1927; May et al., 1931; Buisman, 1933; Banfield and Smith, 1936; Clinton and McCormick, 1936; Wilson, 1965). Abundant mycelia were found in vessels, parenchyma and fibers of inoculated trees, but in naturally infected trees mycelia were uncommon and found only in the vessels (May et al., 1931). However, spores were observed in vessels and fibers, and hyphae were observed in vessels, fibers and parenchyma of twigs taken from diseased trees (Wilson, 1965). Direct penetration of cell walls by *C. ulmi* was not observed. Ouellette (1962) studied sections of wood collected near the site into which spore suspensions had been introduced. He found such abundant mycelia and spores that he attributed blockage of the vascular system to their presence. In addition, he also observed the direct penetration by *C. ulmi* through bordered pits and cell walls. Growth of *C. ulmi* from vessel to vessel or to other cells could account for both the lateral movement of the fungus and the seasonal recurrence of the disease.

Both seasonal recurrence and recovery of diseased American elm have been observed (Walter, 1939; Smucker, 1940; Banfield, 1947). Recurrence varied from 10 to 92 percent in trees with a diameter at breast
height between 1 and 15 in. respectively. Invasion of new xylem by C. ulmi was responsible for the recurrence; where invasion did not occur (in trees of smaller diameter), recovery was evident (Banfield et al., 1947). Twig inoculations made in mid to late summer that resulted in symptoms in the year of inoculation, but in which C. ulmi did not become systemic, rarely wilted during the following season (Smucker, 1937; Banfield, 1948; Neely, 1968). Disease occurred in trees inoculated by S. multistriatus in late spring to early summer; however, later inoculations gave symptoms less frequently or not at all. In apparently healthy trees the fungus could be isolated from feeding wounds after a period of three years (Parker et al., 1941). Attempted isolations from recovered elms in Great Britain have yielded the fungus after a period of up to seven years (Peace, 1960). However, the ability to isolate C. ulmi from recovered American elms declined gradually over a four-year period (Smucker, 1940). A survey of apparently healthy elms also showed the presence of C. ulmi (True and Slowata, 1939). In recovered trees the fungus became confined to those vessels invaded in the year of symptom expression and was buried by subsequent rings of healthy tissue (May, 1934; Buisman, 1936; True and Slowata, 1939; Banfield, 1968).

Xylem discoloration

The discoloration of vascular tissue following infection has been a generally recognized feature of wilt diseases. The "browning reaction" in the conductive vessels and the surrounding xylem parenchyma has recently been shown to be caused by an increase of phenolic substances following fungal invasion of the tissue (Davis and Dimond, 1954; Winstead and Walker,
The discoloration of xylem parenchyma may result from oxidation of phenols to produce melanoid pigments which ultimately spread to other xylem elements or become trapped in occluding gums (Dimond, 1955). The exact mechanism by which the fungus induces the "browning reaction" is unknown. Waggoner and Dimond (1956) have shown that polyphenoloxidases (PPO) are present in healthy tomato shoots but lack the suitable phenolic substrate. They speculated that phenols are liberated from the conjugated form (lignin, glycosides, tannin) by hydrolytic enzymes produced by the invading pathogen. These phenols in turn are oxidized by the PPO of the host tissue to form colored products. Similar increases in concentration of phenolic substances or PPO, or both, have been demonstrated in watermelon (Nishimura, 1959) and tobacco (Sequeira and Kelman, 1962). Studies on phenols of healthy and diseased banana roots indicated that the major phenol, 3-hydroxytyramine, occurred in an unconjugated form, unlike those above, and furthermore was localized within cytoplasmic masses in scattered xylem parenchyma (Mace, 1963). Following infection, discoloration appeared in similarly scattered xylem parenchyma and was diffused by gels into xylem vessels. Further evidence indicated that both fungal and root PPO catalyzed oxidation of the phenol.

Vascular discoloration likewise is found in tree wilt diseases. The discoloration of the vessels of oak infected by *Ceratocystis fagacearum* Bretz has been credited to the presence of dark colored gums (Struckmeyer et al., 1954). Vascular discoloration in *Diospyros virginiana* L. infected by *Cephalosporium diospyri* Crandall has been shown initially to be due to browning of the cytoplasm of xylem parenchyma.
At this early stage of disease development, vessels and fibers showed no discoloration. Later, gums were shown to fill the lumen of the outer vascular elements and intercellular spaces giving the wood the characteristic streaked appearance (Wilson, 1963).

Elm wood, following invasion by \textit{C. ulmi}, similarly undergoes vascular discoloration. This was observed by many early workers (Schwarz, 1922; Wollenweber, 1927; Buisman, 1933; Clinton and McCormick, 1936). Schwarz (1922) found that browning began in the "vascular bundles" and later spread to the other elements of the wood. She also noted that the wood appeared macerated at the stage when massive discoloration began to occur. Other microscopic examination indicated the primary area of browning to be the "large pit vasculars and vascular bundles clogged by resinous bulbs" (Wollenweber, 1927).

More recently, additional histological observations have added to knowledge about vascular discoloration (Kerling, 1955; Ouellette, 1962; Tchernoff, 1965; Wilson, 1965; Gagnon, 1967a,b). Kerling (1955) studied the changes in the diseased tissues of two European elm hybrids. She found that discoloration of the vessel and tracheal walls was the first visible alteration of elm tissue. Browning of the contents of living cells followed. At approximately the same time gum droplets were exuded through the pit membranes and ultimately accumulated within the vessels.

The discoloration in \textit{C. ulmi}-inoculated American elm wood has been observed as early as two days after inoculation (Ouellette, 1962). Disintegration of bordered pits and cell walls was noticed, but only after further disease development. Ouellette (1962) also suggested that many of the early observers looking at gum droplets in reality may have
been observing conidia or "microendospores."

The most recent work on discoloration of xylem tissue of American elm has indicated that the first change to occur following infection was the yellowing of pit membranes (Gagnon, 1967b). Yellowing extended to vessel walls and produced a continuous discoloration, as was also noted by Kerling (1955). The next visible change took place in the living protoplasm near affected vessels. The protoplasm became increasingly granular and the cell contents became first yellow and then brown. The pit membrane also disintegrated, as previously noted by Ouellette (1962). Gum droplets were exuded from parenchyma cells into adjacent vessels, often completely occluding them.

Additional studies noted the distribution pattern of polyphenols in healthy and diseased American elms (Gagnon, 1967b). Healthy elm tissue yielded little reaction to the test for polyphenols, a light color reaction was found in newly formed cells, and a stronger reaction occurred in a few scattered parenchyma cells. In infected elms a strong positive reaction was observed as early as two days after inoculation in cells near the inoculation point. After five days this reaction intensified and was noted in almost all ray and axial parenchyma cells. As infection progressed, the vessels became abundantly filled with phenolic compounds, and these spread into fibers and other vessels until most cells were filled with phenols.

Tests conducted to detect polyphenoloxidase and peroxidase in diseased as well as healthy trees showed the presence of both enzymes. In conclusion, Gagnon (1967b) suggested that elms possess enzyme systems responsible for the oxidation of polyphenols that results in xylem
discoloration.

Additional histochemical tests of elm xylem infected by *C. ulmi* demonstrated other alterations of elm tissue (Gagnon, 1967a). The phloroglucinol-HCl test for lignin showed a more intense reaction in diseased tissue than in healthy elm xylem. Similarly, the Krajcinovic test (Pearse, 1960) for pectin showed intensive staining of the cell walls, including the middle lamella of infected tissue. The stain reaction was limited to the middle lamella in healthy tissue.

Resistance

Research on Dutch elm disease has been oriented primarily toward understanding the nature of the disease in susceptible hosts, and control measures. For this reason little research has been conducted to understand the mechanisms of resistance. Most studies have been involved with the identification of resistance rather than resistance mechanisms. Recently, however, workers concerned with the explanation of resistance have investigated the alteration of both resistant and susceptible host tissue by *C. ulmi*.

Phenolic compounds, discussed in the preceding section, are common in higher plants and especially in woody species, including elms (Metcalfe and Chalk, 1950). These compounds continually have been assigned a rather vague role in disease resistance. A correlation has been shown to exist between the natural phenol level in plants and the degree of disease resistance (Walker and Link, 1935; Lee and Le Tournear, 1958; Patil *et al.*, 1962). Other research has provided evidence indicating that the production or liberation of phenols by plant tissue after infection is
important in disease resistance (Davis et al., 1953; Kuć et al., 1956; Cruickshank and Perrin, 1960; Király and Farkas, 1962). Their part in the host-parasite interaction in Dutch elm disease is unknown.

An interesting comparison of histological responses of the highly susceptible Belgica elm with the responses of the resistant Bea Schwarz elm was made by Tchernoff (1965). He observed initial discoloration (phenol-oxidation) in the vessels with subsequent spread of discoloration from these cells to living cells of the xylem. Discoloration in the highly susceptible Belgica elm was not confined to a restricted portion of the xylem. This discoloration appeared to advance in a tangential direction, including more and more of the earlywood until discoloration encircled the stem. The Bea Schwarz elm, however, contained sharply localized discolored patches consisting of one vessel or a group of vessels. He found in further observations that the spotted pattern was generally found in elms with a high degree of resistance.

The multiplication and transport of spores in xylem vessels of resistant elms were shown to occur at a much lesser rate than in more susceptible species (Elgersma, 1967). Occlusion of vessels by rapid excretion of gums was implicated as the factor limiting spore dispersal in resistant plants. Few vessels became infected above the point of inoculation, and little infection occurred in vessels which had not been infected directly by inoculation. These conclusions are in agreement with the hypothesis of Beckman (1966). He suggested that vascular infections of plants are localized by a three-step mechanism: 1) the screening of mobile cells or spores from the transpiration stream on porous perforation plates and end walls; 2) the occlusion of the infected
portion of the vessels by gels secreted by the stimulated parenchyma cells; and, 3) the occlusion of the infected portion of the vessel by tyloses. The rapidity of the host responses mentioned above determines the degree of resistance.

An analysis of the sap constituents of various elm species has shown marked differences between resistant and susceptible species (Elgersma, 1967; Singh and Smalley, 1969). Proline and $\alpha$-amino-$n$-butyric acid were present in considerable amounts in the sap of resistant species in contrast to only trace amounts in the sap of susceptible elms (Singh and Smalley, 1969). Sucrose was the major sugar in the sap of resistant species, while susceptible species contained both sucrose and fructose in approximately equal amounts. Elgersma (1967), however, did not detect differences in sugars between resistant and susceptible clones. The total concentrations of amino acids and ammonia were higher in resistant species than in susceptible species (Elgersma, 1967; Singh and Smalley, 1969). Although the exact relationship between proline content of the xylem sap and resistance is unknown, the presence of proline was always correlated with resistance.

In comparisons of Siberian elms (U. pumila L.) and American elms (U. americana) a difference was observed in resistance toward physiological wilt (Dimond et al., 1949). Variation in susceptibility to water shortage corresponded to variation in susceptibility to C. ulmi. Another study considered the differences in transpirational rates of artificially inoculated U. americana and U. pumila (Roberts, 1966). The transpiration in both increased 10 percent the week after inoculation, but after four weeks transpiration rate of U. americana dropped to 21 percent of the controls. U. pumila decreased to 85 percent of that of the controls after
four weeks. Roberts (1966) suggested that failure of *U. pumila* to develop foliar symptoms may be attributed in part to its natural drought resistance.

The preceding studies concerned with resistance and spore dispersal hint at the possible relationships between vascular anatomy and resistance. Wood structure, however, was shown to have no effect on the movement of spores through either resistant or susceptible European elms (Buisman, 1936). Subsequent investigators (Dimond et al., 1949) found no difference in vessel length or vessel diameter between *U. americana* and *U. pumila*. They agreed with Buisman that resistance cannot be accounted for on anatomical grounds. Comparisons between the vascular anatomy of *U. pumila* and *U. americana* by Pope (1943), however, suggested an anatomical relationship to resistance. His observations indicated that *U. pumila* had a short susceptibility period because of its early initiation and subsequent production of latewood. In *U. americana* latewood production was delayed. He further characterized latewood by the lack of interconnections between vessels and groups of vessels. He concluded that the fungus was isolated in that portion of the wood where inoculation was made.

Investigators have noted that the general physiological condition of the European elms is important for the development of external disease symptoms. In general, they have indicated that vigorously growing trees showed more disease symptoms than those which did not exhibit such vigor (Buisman, 1935; Went, 1954, Heybroek, 1957; Peace, 1960). Similarly, Canadian workers testing seedling populations reported that large vigorously growing American elms reacted more severely to inoculation than smaller less vigorous trees (Ouellet and Pomerleau, 1965). They also noted that external disease symptoms were stimulated when they kept the soil
moist. Other observers of American elms have reported that trees grown on soils with a high water-holding capacity were more susceptible than those growing on soils with a low water-holding capacity (Kais et al., 1962). Vascular discoloration was greater on plants watered daily than on those watered every fifth day.

Other related studies have demonstrated that young seedlings of American elm inoculated shortly after transplanting remained symptomless (Smalley, 1963). Discoloration of the xylem was similarly limited. These findings can be related to Beckman's (1958) conclusions that Dutch elm disease can be retarded by treatments that slow tree growth.

There has been further evidence that young seedlings resist the disease (Caroselli and Feldman, 1951; Went, 1954; Heybroek, 1957). Elm seedlings appeared to become progressively more susceptible with age. Other workers have disputed the concept of "youth or juvenile" resistance (Arisumi and Higgins, 1961; Smalley, 1963; Tchernoff, 1965).

A periodicity of susceptibility of European elm to C. ulmi also has been observed. In 1960 and 1961, a period of extreme susceptibility began 45 and 52 days after bud break, respectively. The period of susceptibility extended for 30 days in 1960 and only 13 days in 1961; however, in both years only during a period of one week could successful inoculation be assured (Tchernoff, 1965). Smalley (1963), studying the seasonal fluctuations of disease in American elm seedlings, correlated the decline in susceptibility with the cessation of terminal growth.

Tyloses

The occurrence of tyloses in vessels of chestnut wood was noted as
early as 1675 by Malphigi (Gerry, 1914). Tyloses have since been observed in the vessels of innumerable vascular plants (Gerry, 1914; Chattaway, 1949; Esau, 1965).

Few studies have been conducted to determine what factors are responsible for tylose formation. Chattaway (1949) correlated the ability to form tyloses in the heartwood of many tree species with the width of the aperture of the pits extending from vessel to ray cells. She concluded that a pit aperture of less than 10μ prevented tylose formation. The presence of air within the vessels has been suggested as the cause of tylose development (Klein, 1923). Decomposition products from traumatized cells also have been thought to stimulate tylose production (Haberlandt, 1923). The results of other research have shown that tyloses can result from wounding (Block, 1952; Meyer, 1967) or from water stress (Zycha, 1948). Toxic metabolic by-products which have accumulated within parenchyma cells have been thought to stimulate tylose formation in the inner sapwood (sapwood-heartwood boundary) (Stewart, 1966). The toxic metabolites are thought to originate within the parenchyma cell and also to diffuse from the cambium to the cells of the inner sapwood. Stewart (1966) further speculated that the concentration of toxic substances is reduced by the expansion of the parenchyma cells during tylose formation. This expansion permits the cells to remain alive.

Reports of tylose formation after vascular invasion by wilt-disease organisms are numerous. In oak wilt, plugging of vessels by tyloses and gums is extensive and thought to be the cause of wilting (Struckmeyer et al., 1954). Tyloses have also been reported in wilt
diseases of tobacco (Powers, 1954), hops (Talboys, 1958), grapes (Esau, 1948), bananas (Beckman et al., 1962), watermelons (Sleeth, 1933) and sweet potatoes (McClure, 1950). The occurrence of tyloses was noted less commonly in wilt diseases of tomato and mint (Dimond, 1955).

The plugging of vessels following vascular invasion by wilt pathogens has caused further speculation about the possible mechanisms of tylose formation. Weakening of pit membranes by pectic enzymes of the parasite was thought responsible (Waggoner and Dimond, 1955). High concentration of indole acetic acid (IAA) has been demonstrated in plants suffering from vascular invasion (Pegg and Selman, 1959; Sequeira and Kelman, 1962). Sequeira and Kelman (1962) have concluded that pathogen-produced IAA probably causes tylose formation. Studies of banana likewise have indicated a possible connection between IAA and the development of tyloses (Mace and Solit, 1966).

The loosening of inter-polymer bonds in the amorphous cell wall matrix has been suggested to provide the plasticity essential for the growth of parenchyma cells that results in tylose formation (Beckman, 1969). The results of this research indicated that marked diurnal changes in pH occur in the vascular tissues of infected roots, and these changes are in turn probably related to CO₂ accumulation and possible CO₂ fixation associated with an infection-induced, high rate of respiration. The amplitude of the diurnal changes in pH was shown to be sufficient to permit swelling of cell walls in the presence of the accumulated CO₂ and/or carboxylic acids.

The occlusion of the conducting vessels of C. ulmi-infected elm by
Tyloses has been similarly observed (Schwarz, 1922; Wollenweber, 1929; Broekhuizen, 1929; Buisman, 1933; Clinton and McCormick, 1936; Kerling, 1955; Beckman, 1958; Ouellette, 1962; Wilson, 1965; Tchernoff, 1965). Schwarz (1922) stated that the first changes in appearance of infected elm wood were in the form of what she calls "bladder-like tyloses" found in the "vascular bundles." Tyloses were shown to occur in diseased elm wood even when the transpiration stream remained unbroken (Broekhuizen, 1929). These findings were in conflict with the theory then generally accepted for tylose formation.

In other studies, tyloses were observed only in vessels in the proximity of other vessels containing fungal material (Kerling, 1955). Furthermore, it was speculated that high "toxin" concentration in vessels containing fungal material inhibited tylose formation; from this center of high "toxin" concentration, a decreasing gradient of toxin resulted in the stimulation of tyloses in adjacent vessels. Development of abundant tyloses through pits with apertures of less than 10μ was also observed, disputing Chattaway (1949).

The tyloses formed in artificially inoculated American elms were thought to be insufficient to cause wilting (Ouellette, 1962); however, other research on tylose development in naturally infected American elms suggests that there is sufficient production of tyloses to incite wilting (Wilson, 1965).

Related Ultrastructural Studies

To this date there have been no electron microscopic studies of diseased elm tissue. However, several light and electron microscopic
studies have provided information that has been useful in the interpretation of the electron microscopic observations.

**Fungal involvement**

The bacteria and fungi which induce vascular wilting are facultative parasites. They must invade and colonize the host before they can produce wilting. Husain and Kelman (1959) suggested that the nutrients required for this colonization were derived from the cell walls of the vascular tissue and from the reserve food within the xylem parenchyma. They further state that the resultant breakdown of tissue was so limited initially that no microscopic symptoms of the dissolution of tissue were evident. Wilt diseases of trees and woody shrubs probably shows less apparent damage because of considerable lignification of the cell walls of the vascular tissue. This situation may be one extreme while the bacterial wilts of succulent herbaceous annuals are the other. In this situation, cells of the xylem, phloem, pith and cortex frequently were rapidly disintegrated, resulting in a soft decay of localized areas within the stem (Husain and Kelman, 1958).

Few generalizations can be made about the actual methods used by fungi to attack host cells. However, the fungi can be separated into two groups by their effect upon host cell tissues (Wheeler, 1968). The facultative parasites rapidly kill the cells they invade or are in contact with, whereas the cells colonized by obligate parasites are not killed. The formation of haustoria within host cells is associated with this type of parasitism.

Even though studies concerning the host-pathogen interaction
recently have been extended to the electron microscopic level, the relatively few investigations dealing with such interactions have concentrated on haustoria. A generalized concept of haustorial structures has been presented by Brackner (1967). Other workers have proposed a specific host-parasite system from electron micrographs of *Raphanus sativus* L. infected by *Albugo candida* (Lév.) Kunze (Berlin and Bowen, 1964).

The protoplasmic structure of obligate parasites is not significantly different from that of facultative parasites (Brackner, 1967). Other than these general cytoplasmic observations little attention has been paid to the involvement of facultative parasites in disease situations. Results of a recent ultrastructural study of *Pyrenochaeta terrestris* (Hansen) Gorenz, J. C. Walker & Larson infected onion roots showed that the fungus penetrated primarily or entirely by dissolution of host-cell walls rather than by pressure of fungal cells (Hess, 1969). This situation was suggested by the presence of a zone of greater electron density about the hyphal tip of the penetrating fungus. Appressoria commonly associated with penetration by force (Dickinson, 1960) also were absent. Additionally, Hess (1969) observed that the fungus caused the cytoplasmic disruption of one to several cells in advance of the fungus. The production of pectolytic and cellulolytic enzymes by *P. terrestris* has been demonstrated by Horton (1964). These enzymes have been discovered both *in vivo* and *in vitro* and thus this fungus is capable of producing the observed dissolution.

For fine-structural information on the colonization of woody tissues by facultative parasites it is necessary to consider the work with
wood-decay fungi. Cowling (1965) suggested that two major types of enzymatic activity on wood cell walls by wood-destroying fungi are distinguishable: 1) localized dissolution of cell walls at points of contact with the fungal hyphae; and 2) generalized dissolution of cell-wall substance at some distance from the hyphae of the organism. The production of so-called bore holes typifies the first type of cell-wall decay. Proctor (1941) concluded from light microscope studies of fungal hyphae in the process of forming bore holes, that penetration was accomplished by the enzymatic mechanisms rather than by application of mechanical forces. Similar conclusions were drawn from the electron microscopic study of spruce and sweet gum infected by Polyporus versicolor L. ex Fr. (Cowling, 1965). However, Cowling (1965) additionally noted that the disintegration of cell-wall substances also occurred on the surfaces of the lumen of wood cells where no contact with fungal hyphae was involved.

The Dutch elm disease fungus, Ceratocystis ulmi, has been shown to produce low levels of PG in culture (Beckman, 1956). Additionally, weak cellulase activity has been noted in cultural filtrates of C. ulmi (Beckman, 1956; Husain and Dimond, 1958). Therefore, C. ulmi apparently possesses the enzyme systems that are capable of tissue disintegration or colonization such as those described above.

Tannin accumulation

The term "polyphenol" has been used to refer to a wide variety of aromatic compounds. Earlier literature referred to the same group of compounds as "tannins." In studying the histological changes that occur upon infection of elm by C. ulmi the role of polyphenols must be considered. The literature abounds with chemical characterizations of phenolic
compounds and their function in cell metabolism but little attention has been paid to their histological development within tissues.

The accumulation of phenols has been shown to accompany the transition of sapwood to heartwood (Frey-Wyssling and Bosshard, 1959). The oxidation of accumulated phenols progresses in many tree species to form the dark brown pigments responsible for the darkened heartwood. This formation of tannins has been observed to occur within the cell vacuoles (Bailey, 1954; Bosshard, 1968). According to Chattaway (1952) the ray cells of the intermediate band between sapwood and heartwood display an intensified metabolism characterized by the formation of tyloses, production of phenols, and disappearance of starch.

Stained or discolored wood can form in the living sapwood as the result of injury by wounding or of infection by parasitic organisms. Such a reaction is common to woody-plant wilt diseases including Dutch elm disease and likewise has been suggested to result from phenol oxidation (Cagnon, 1967a). Although the phenols of woods affected by these forms of injury have been shown to be chemically different from those of normal heartwood, it appears that the site of their accumulation is similar (Hillis, 1968).

The vacuole, one of the most characteristic features of the differentiated plant cell, is the cytoplasmic organelle that has received the least attention (Esau, 1963). Esau (1963) further states that the vacuoles of differentiated plant cells frequently contain tannin. Most fine-structural studies have accepted the presence of vacuolar tannin, and only a few studies have dealt with its origin and accumulation. The formation of phenolic compounds in the ray parenchyma of Eucalyptus has
been noted (Wardrop and Cronshaw, 1962). The phenolic substances appeared to originate within a structure limited by a double unit membrane. Phenolics were found in vesicles in an organelle similar to a chloroplast. Additional observation showed that the vesicles arose from the dissolution of the starch grains contained within the organelle. The ultrastructural study of Eastern white pine needles infected by Cronartium ribicola Fischer likewise indicated that polyphenolic compounds were enclosed by vacuolar membrane in healthy as well as diseased tissue (Boyer and Isaac, 1964).

Tylose formation

The ultrastructural literature on tyloses is concerned primarily with the mature tylose wall. Examination of several American hardwoods showed that mature tylose walls are composed of both primary and secondary wall layers, indicated by the corresponding random and parallel microfibrillar orientations (Koran and Coté, 1964; 1965). A substance resembling the middle lamella was noted between contiguous tyloses or between tyloses and the vessel wall. Simple intertylosic pitting was also observed. Other ultrastructural observations have shown that a layer (called the protective layer by Schmid (1965)) adjacent to the cell wall and lining the lumen of the ray cell enlarges to form the tylose (Foster, 1964).

The ultrastructural ontogeny of traumatic (wound) tyloses in Quercus alba L. was investigated by Meyer (1967). His observations indicated that the parenchyma cell and the tylose which develops from it are continuous and are bounded by a common cell wall. The parenchyma cells
that he refers to in his observations are ray parenchyma. Sections through the vessel-parenchyma pit membrane revealed it to be less electron dense under his staining techniques than either the primary or secondary cell wall. Further observations suggested the density of the intertracheary pit membranes were similar to that of the vessel-parenchyma membranes. Staining with potassium-permanganate indicated that neither of these membranes was lignified; however, the interparenchymatous pit membranes stained intensively.

A "protective layer," as previously described but not named by Foster (1964), was situated within the parenchyma cell adjacent to the vessel-parenchyma pit membrane (Schmid, 1965). Meyer and Coté (1968) observed that the tylose wall arises from the protective layer so that after tylose formation the tylose cell wall and the protective layer are continuous. The pit membrane and the protective layer are of similar electron density and could not be distinguished. The protective layer was described as microfibrillar in nature and extended for varying distances around the parenchyma cell lumen. Parallel microfibril orientation, similar to that of the secondary cell wall, was noted.

Meyer (1967) further noted the degradation of vessel-parenchyma pit membrane prior to tylose formation. As the tylose growth is initiated the protoplast expands and forces the protective layer and the pit membrane into the vessel lumen. This enlargement causes the rupture of the degraded pit membrane. The microfibrils that compose the pit membrane separate so the membrane appears to be dispersed. He suggests that the initial degradation of the pit membrane probably destroys the
bonding between the microfibrils.

The parenchyma cell vacuolates as the tylose forms. At maturity the cytoplasm is appressed against the cell wall by the large vacuole that fills nearly the entire volume of the tylose. The volume of cytoplasm apparently remains unchanged during tylose formation and maturation.
MATERIALS AND METHODS

Selection of Host Material

Three field-grown elm selections were chosen for this study because their variable susceptibility to *Ceratocystis ulmi* was known. All trees were located on the Iowa State University Horticulture Farm. *Ulmus pumila* L. var. Chinkota was the least susceptible selection used (Figure 1). To obtain enough branches, two trees of this selection were utilized for both the 1967 and 1968 studies. The Chinkota elms represented a seed-source selection and are not clonal material (Collins, 1955); however, both trees were similar in stature, age and dbh (9 in.). *Ulmus americana* was the most susceptible host. A single tree was chosen for the preliminary 1967 study; a second tree (Figure 2), similar in stature to the other selections, was utilized for the 1968 phase of the investigation. The first became noticeably diseased and consequently necessitated the choice of another tree for the second year's study. *Ulmus carpinifolia* Gleb. 'Christine Buisman,' a Dutch clonal selection (#24) from Madrid, Spain (Figure 3) was intermediate in susceptibility between the other two selections. It should be noted that this clone is much closer to the Chinkota elm in its resistance than to *U. americana*. Two trees of this selection also were used for the 1967 and 1968 studies; both were of similar age and dbh (10 in.).

A preliminary study was conducted during the summer of 1967. The objectives were to determine fungal movement within each respective host and to note host responses, if any, incurred as a result of wounding during the inoculation procedures. These data were used as criteria for the 1968
Figure 1. *Ulmus pumila* var. Chinkota. Siberian Elm. A seed-source selection from South Dakota known for its winter hardiness.
Figure 2. *Ulmus americana*. American elm. A single field grown elm used for the 1968 studies.
Figure 3. *Ulmus carpinifolia* 'Christine Buisman'. Buisman elm. A Dutch clonal selection (#24) from Madrid, Spain.
phase of the investigation.

Inoculum

Fourteen-day-old mixed shake cultures of *C. ulmi* were used for the production of inoculum. The cultures were grown in 50 ml of a modified Zentmeyer's liquid medium (Ouellette and Gagnon, 1960). Five fungal isolates for shake culture were obtained from recent cultures of *C. ulmi* taken from *U. americana*.

Three shake cultures were mixed together and subdivided into four centrifuge tubes, the excess spore suspension being discarded. The tubes were centrifuged at 5,000 rpm for 10 minutes at room temperature. The supernatant was decanted and the spores were washed three successive times with sterile distilled water. The same centrifuging procedure was followed after each washing. The spore pellets were diluted with 100 ml sterile distilled water. Immediately after dilution the spore concentration of the suspension was determined. This suspension was used immediately for inoculation. The conidial suspension used for the 1967 inoculations contained approximately $1.2 \times 10^4$ spores per ml; for the 1968 inoculations, the suspension contained approximately $10^6$ per ml.

Inoculations and Sampling - 1967 Series

Two-year-old twigs of each host were selected for inoculation. This age twig duplicated the site of natural fungal inoculation by the smaller European elm bark beetle. A droplet of inoculum was placed on the twig at the desired point of inoculation. The twig was pricked with a dissecting needle to a depth immediately below the cambial layer (Figure 4, 5).
Figure 4. Inoculation of a two-year-old elm twig

Figure 5. Point of inoculation tagged for subsequent collection
The inoculum was drawn into the twig upon the removal of the needle. Control twigs were inoculated in a similar manner substituting sterile distilled water for the spore inoculum.

On June 3, 1967, 108 twigs were inoculated at random. The two selections of two trees each were treated as a single tree. Collections of inoculated twig material were made at 1/2, 1, 2, 3, 5, 7, 9, 12 and 15 days after inoculation. At each collection period, three replications of inoculated twigs plus a single inoculated control twig were collected for each of the tree selections (Table 1).

Each twig was cut into five segments, three segments distally and two basally from the inoculation point. A portion of each twig segment was cultured and a portion preserved in FAA solution (Sass, 1958) (Figure 6). Twig segments were cultured to obtain information about fungal movement. Both culturing and fixation were conducted in the field; for this reason, the segments were plated on 2-percent water agar to reduce growth of fungal contaminants.

Inoculations, Sampling and Embedding - 1968 Series

The data collected from the 1967 studies were used as criteria for the second series of inoculations during the spring of 1968. This material was in turn to be used for the subsequent light- and electron-microscopy study.

The same procedures for inoculation were followed. Twigs were inoculated on June 3, 1968. Collections of inoculated material were made after 1, 3, 5, 9 and 12 days. For each collection, two branches per selection were taken during the morning of the five sampling dates.
Table 1. Inoculation procedures

<table>
<thead>
<tr>
<th>Selections</th>
<th>Inoculation date</th>
<th>No. of twigs inoculated per selection</th>
<th>No. of <em>C. ulmi</em> inoculated twigs collected on each date</th>
<th>No. of control (H&lt;sub&gt;2&lt;/sub&gt;O inoculated) twigs collected on each date</th>
<th>No. of collection dates</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>U. americana</em></td>
<td>June 3, 1967</td>
<td>36</td>
<td>3</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td><em>U. carpinifolia</em></td>
<td>June 3, 1967</td>
<td>36</td>
<td>3</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td>'Christine Buisman'</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>U. pumila var.</em></td>
<td>June 3, 1967</td>
<td>36</td>
<td>3</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td>Chinkota</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>108</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>U. americana</em></td>
<td>June 3, 1968</td>
<td>12</td>
<td>2</td>
<td>2</td>
<td>5&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>U. carpinifolia</em></td>
<td>June 3, 1968</td>
<td>12</td>
<td>2</td>
<td>2</td>
<td>5&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>'Christine Buisman'</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>U. pumila var.</em></td>
<td>June 3, 1968</td>
<td>12</td>
<td>2</td>
<td>2</td>
<td>5&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>Chinkota</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>36</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>1</sup>Control twigs collected on fifth date only.
On the final collection date two control branches were cut from each selection (Table 1). The material was transported immediately to the laboratory for fixation and embedding. The nature of the fixation process required laboratory conditions. For this reason, branch samples were cut two to three feet basally from the point of inoculation in an effort to avoid an immediate wounding response in the area of microscopic examination. Excess branch material was trimmed from each twig before the preparation of that twig for fixation.

The area of twig to be studied was immersed completely in phosphate buffer (Lillie, 1954) held at approximately 4°C. The initial transverse cut was made approximately 3 in. distal to the point of inoculation. Subsequent transverse sections were made as thin as possible with a double-edged razor blade toward the inoculation point (Figure 7). The phosphate buffer was then drained from the thin sections, and the sections were fixed for one hour at 4°C in a 2-percent aqueous osmium tetroxide solution mixed 1:1 with buffer (Millonig, 1961).

The procedure for dehydration and the specific process of embedding in Epon (Luft, 1961) or Araldite-Epon (Anderson and Ellis, 1965) are outlined in Appendix A. Material collected on sampling dates 1, 3 and 5 were embedded in Epon; subsequent collections were embedded in Araldite-Epon. The Araldite-Epon medium facilitated the cutting of 1- to 2-micron sections (utilized for light microscopy) with glass knives.

Light Microscopy

Epon and Araldite-Epon sections 1- to 2-microns thick were prepared for light microscopy. They were cut with a glass knife on an LKB
Figure 6. Branch sampling procedures used during the 1967-series of inoculations

Figure 7. Branch sampling procedures used during the 1968-series of inoculations
Ultratome III ultramicrotome. The glass knives were modified by using tape and dental wax to provide a water surface upon which to collect the sections. The sections were picked up with an "eye-lash" microtool and collected on a slide in a drop of distilled water. The slide was heated to speed drying. The sections were then mounted permanently in piccolyte and observed by phase-contrast light microscopy. Additional slides were stained in toluidine blue or aqueous safranin 0 prior to permanent mounting (Feder and O'Brien, 1968). This staining process was adopted to improve contrast for photographic purposes. The methods described above limited the size of the cutting surface that could be sectioned. However, the improved fixation and thinness of sections obtained by this method greatly outweighed this limitation and proved superior to previous techniques of embedding woody tissue in paraffin and celloidin.

The comparison of related cells from light to electron microscopy was extremely important in this study. When an area of interest was located, the specimen face was trimmed to include cells pertinent to the study; further sectioning for electron microscopy then proceeded.

Photographs were taken on a Zeiss photomicroscope using an automatic 35-mm camera. Phase-contrast and bright-field photomicrographs were taken on Kodak Panatomic-X film.

Electron Microscopy

Sections of Epon- and Araldite-Epon-embedded material for electron microscope studies were cut with a Dupont diamond knife on an LKB Ultratome III ultramicrotome. Section thickness ranged from 60-90 millimicrons as indicated by diffraction colors. Quality of the
Araldite-Epon sections was improved by expanding the sections with xylene vapors (Anderson and Ellis, 1965). The sections were picked up on carbon-coated, formvar-supported 100-mesh copper grids. Thin sections were observed either unstained or stained in methanol uranyl acetate (Stempak and Ward, 1964) or lead citrate (Reynolds, 1963).

Specimens were observed on an RCA EMU-3F electron microscope operated at 50 kv. Direct magnifications of approximately 1500 to 20,000 X were photographed on Cronar Ortho-s-Litho film. These films were developed in Kodak D-19 developer for 1.5-2 minutes at 68° F. All negatives were enlarged and printed on Kodabromide F single-weight paper.
RESULTS AND OBSERVATIONS

1967 Series of Inoculations

The culture data obtained from the sampling of inoculated branches are given in Table 2. A positive culture was assumed to mean that the fungus had moved into that section of the twig. The differences in fungal movement within the two resistant varieties were negligible. There appeared to be as much variation within each of these selections as between them. The data obtained from the culturing of the American elm branch samples presented a different situation. During the same length of time the fungus moved farther from the point of inoculation. Each of the replications collected at the various sampling dates substantiated this relationship.

Positive culture results were obtained within each selection at a position approximately 3 inches distal to the point of inoculation on the first day after inoculation. A wound response originated at the point of inoculation in both control and inoculated elm and this response appeared as a dark streak in the xylem tissue running longitudinally in both directions from the inoculation point. However, light-microscopic examination of the control material revealed that the response did not extend to a distance 3 inches from the inoculation site. Therefore this position was selected as the site for the following year's sampling.

1968 Series of Inoculations

Light microscopic observations

Several aspects of disease development were first noted during
Table 2. Culture data from 1967 series of inoculations, representing three replications of *C. ulmi*-inoculated twigs and a single control twig

<table>
<thead>
<tr>
<th>Date and selection (Days after inoculation)</th>
<th>Basal</th>
<th>Terminal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Basal</td>
<td>Terminal</td>
</tr>
<tr>
<td></td>
<td>4&quot;1</td>
<td>2&quot;</td>
</tr>
<tr>
<td>June 3 (1/2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 - <em>U. americana</em></td>
<td>---2</td>
<td>-</td>
</tr>
<tr>
<td>2 - <em>U. carpinifolia</em> 'Christine Buisman'</td>
<td>---</td>
<td>-</td>
</tr>
<tr>
<td>3 - <em>U. pumila</em> var. Chinkota</td>
<td>---</td>
<td>-</td>
</tr>
<tr>
<td>June 4 (1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 - <em>U. americana</em></td>
<td>---</td>
<td>+</td>
</tr>
<tr>
<td>2 - <em>U. carpinifolia</em> 'Christine Buisman'</td>
<td>---</td>
<td>++</td>
</tr>
<tr>
<td>3 - <em>U. pumila</em> var. Chinkota</td>
<td>---</td>
<td>-</td>
</tr>
<tr>
<td>June 5 (2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 - <em>U. americana</em></td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>2 - <em>U. carpinifolia</em> 'Christine Buisman'</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>3 - <em>U. pumila</em> var. Chinkota</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>June 6 (3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>i - <em>U. americana</em></td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>2 - <em>U. carpinifolia</em> 'Christine Buisman'</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>3 - <em>U. pumila</em> var. Chinkota</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

1. For each position, first group represents the three *C. ulmi*-inoculated twigs; second group represents control.

2. + equals positive culture for *C. ulmi*; - equals negative culture.
Table 2 (Continued)

<table>
<thead>
<tr>
<th>Date and selection (Days after inoculation)</th>
<th>Basal</th>
<th></th>
<th>Terminal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4&quot;</td>
<td>2&quot;</td>
<td>3&quot;</td>
</tr>
<tr>
<td>June 8 (5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 - U. americana</td>
<td>+++</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>2 - U. carpinifolia 'Christine Buisman'</td>
<td>+++</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>3 - U. pumila var. Chinkota</td>
<td>+++</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>June 10 (7)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 - U. americana</td>
<td>+++</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>2 - U. carpinifolia 'Christine Buisman'</td>
<td>+++</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>3 - U. pumila var. Chinkota</td>
<td>+++</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>June 12 (9)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 - U. americana</td>
<td>+++</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>2 - U. carpinifolia 'Christine Buisman'</td>
<td>+++</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>3 - U. pumila var. Chinkota</td>
<td>+++</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>June 15 (12)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 - U. americana</td>
<td>+++</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>2 - U. carpinifolia 'Christine Buisman'</td>
<td>+++</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>3 - U. pumila var. Chinkota</td>
<td>+++</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>June 18 (15)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>1 - U. americana</td>
<td>+++</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>2 - U. carpinifolia 'Christine Buisman'</td>
<td>+++</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>3 - U. pumila var. Chinkota</td>
<td>+++</td>
<td>-</td>
<td>+++</td>
</tr>
</tbody>
</table>
light microscopic examination of the diseased tissue. The presence of fungal material, the accumulation of phenolics and the development of tyloses were observed in the three selections. The initial reaction of the three selections to _C. ulmi_ appeared to be similar and synchronous.

Light-microscopic aspects of the phenomena noted above will be discussed briefly; a greater emphasis will be placed on fine-structural observations.

**Fungal material** The first day after inoculation the fungus was present in vessel elements (Figures 12, 13). Conidia were observed most frequently about the periphery of the xylem vessel (Figures 13, 14, 19, 20, 22, 23, 27), but occasionally the entire vessel was filled with spores (Figures 12, 15, 17, 24). The conidia appeared to be confined to a single vessel or a small group of vessels in both resistant selections (Figures 12, 13, 15, 19, 22, 24). However, the examination of American elm tissue frequently indicated that conidia were found in several vessel elements (Figures 14, 17, 23). These elements were not always contiguous.

The conidia were similar to those observed in shake culture. They likewise appeared to undergo budding, which was also viewed in culture. Five days after inoculation short hyphal branches were observed growing through intertracheary pit membranes (Figures 29, 35). In no case was direct cell-wall penetration discernible with the light microscope.

**Accumulation of phenolics and discoloration** The region of discoloration, clearly visible to the unaided eye, was not distinct in the 1-micron sections. Darkening of the intertracheary pit membranes was observed one day after inoculation (Figures 12, 13, 48). The
alteration was limited to those membranes within the region that I have termed the "reactive zone." Similarly, a darkening of the contents of both ray and vasicentric parenchyma cells was seen after one day (Figures 11-13). Tests to specifically characterize these compounds were not conducted. However, their positive reaction to osmium tetroxide identified them as phenolic compounds. This increase in phenolic accumulation coincided with a decrease of starch grains which were abundant in healthy tissue (Figures 8-10). The accumulation of phenolics intensified in subsequent collections until the contents of virtually all xylem parenchyma within the reactive zone were discolored (Figures 14-25). Phenolic products often accumulated within tyloses (Figures 16, 18-20, 22, 25). However, this was not as consistent a feature as phenol accumulation within parenchyma.

Twelve days after inoculation the material became increasingly difficult to section. The wood was brittle and often fractured before a complete section was obtained.

Tyloses Tylose initiation occurred rapidly within the infected host tissue of the three selections. Several vessels within each selection were completely occluded one day after inoculation (Figures 11-13). The development of tyloses then became more extensive in adjacent vessels and other vessel groups (Figures 14-25). The first site of tylose formation was in vessels adjacent to those containing fungal material, but in no case were tyloses ever observed within vessels containing fungal

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1 This term was used to designate the area of host tissue viewed in cross section that had undergone alteration as the result of infection (Figure 5).
material (Figures 11-25). The vessel or vessel groups containing the fungus served as the locus from which tylose initiation began. As infection progressed a greater number of vessels were occluded by tyloses (Figures 11-25). The spread in tylose formation occurred laterally in both directions from this fungal locus. However, within the resistant selections, _U. pumila_ var. Chinkota and _U. carpinifolia_ 'Christine Buisman', fewer vessels became involved in tylose formation, so that the area of tylose initiation remained almost constant by the fifth day after inoculation (Figures 18, 19). By nine days after inoculation, tylose development in _U. americana_ had involved nearly two times the area of xylem tissue as the resistant varieties (Figures 20-22). Tylose formation was always limited to the second year's growth.

Thickening of the tylose wall was occasionally observed (Figures 17, 23-25). Simple intertylosic pitting likewise was observed within mature tyloses and is clearly seen using the electron microscope (Figure 63).

**Electron microscopic observations**

Electron microscopic observations of the phenomena associated with disease development appeared similar in the selections studied. For this reason, the aspects of disease development are discussed without specific reference to selection. The selections used for each illustration are given in the figure legends.

**Fungal relationships** Observations of the fungus-host relationships in diseased elm were improved by the increased resolution of the electron microscope. Numerous conidia were evident in nearly all sections prepared for electron microscopy (Figures 27, 28). Less
frequently short hyphal filaments were noted (Figures 30, 31, 34, 36, 37). The cytoplasmic components of hyphal filaments included mitochondria, endoplasmic reticulum (ER), ribosomes and osmiophytic granules (Figures 31, 34, 36, 37). Dictyosomes were not evident and nuclei often were poorly defined (Figures 31, 33). Lomasomes, highly controversial fungal organelles, were never observed. Septation of the hyphae was evident and when cut in median-section, each septum appeared as a simple plate with a central pore (Figures 30-32). Vacuolation occasionally was observed within hyphal filaments (Figure 37).

The cytoplasmic components of the conidia were less distinguishable, undoubtedly because of poor fixation, so that frequently the cytoplasm appeared granular and of various electron densities (Figures 27, 28). For this reason no conclusions could be drawn about cytoplasmic differences between hyphae and spores. Occasionally wall structure was seen within the existing conidia suggesting that these spores were budding (Figure 32), for an internal wall was not evident when conidia germinated to form hyphal filaments (Figures 30, 31).

Often it was impossible to distinguish conidia from hyphae. The thinness of the sections and the various angles of sectioning resulted in different shapes and sizes of fungal material so that a cross section of a hyphal filament sometimes looked like a spore.

The penetration of cell walls, intertracheary pit membranes and vessel-parenchyma membranes by the germinating conidia and hyphal filaments was observed five days after inoculation (Figures 30, 31, 33, 36-41). Commonly, conidia that were closely appressed to the vessel wall had germinated and directly penetrated the cell wall (Figures 38-40). The
penetration peg of conidia was of a lesser diameter than the spore from which it arose (Figures 38-40). Once wall penetration was accomplished the hypha expanded (Figure 38). During hyphal penetration of cell walls a similar constriction occurred. In either case, the penetration peg was surrounded by an area of greater electron density (Figures 38-41). In median section this area was noticeably larger at the tip of the penetrating fungus (Figure 39). The subsequent hole resulting from penetration therefore appeared larger than the diameter of the penetration peg. In no case was there any indication of force, characterized by the formation of appressoria (Dickinson, 1960), exerted by the fungus during the penetration process.

Hyphae grew through the intertracheary pit membrane but without evidence of a zone of greater electron density about the penetrating fungus (Figures 30, 31, 36, 37, 41). During penetration, the hyphae conformed to the shape of the double-bordered pit walls and apparently had little immediate influence upon them since no change in their electron density was evidenced (Figures 31, 36, 37, 41).

Occasionally a different situation was observed in which the fungus entered the bordered pit through the pit aperture and then directly penetrated the secondary cell wall on the opposite side of the aperture (Figure 41). Thus the growth of the fungus seemed quite irregular, except that intertracheary pit membranes were penetrated more frequently than were secondary cell walls.

Hyphae also penetrated ray and vasicentric parenchyma cells through the vessel-parenchyma pit membrane (Figures 33, 34). Penetration and colonization always was viewed after the cytoplasmic breakdown of these
cells. An area of greater electron density similar to that associated with fungal penetration of the secondary cell wall was not evident in this situation.

**Phenol accumulation** The discoloration of host tissue noted during the light phase of this study was further examined at the ultrastructural level. The electron density of the intertracheary pit membranes increased, so that they appeared completely darkened (Figures 36, 37, 39, 49). In contrast, the membranes of the control material were of a density similar to that of the embedding medium (Figures 42, 43). The darkening of the membrane furthermore was accompanied by the superficial alteration of the cell-wall surface included within the bordered pit (Figures 30, 36, 37, 39, 49). Frequently the darkened membranes appeared granular, and particles of similar density were associated with the membranes (Figure 49). In contrast to the observed alteration of the intertracheary pit membrane no change in the electron density of the vessel-parenchyma pit membrane was seen. The electron density of the cell wall did not change during this study.

Abundant phenolic compounds accumulated within the vacuoles of the xylem parenchyma (Figures 50-53). Two methods of accumulation were noted. The majority of the phenolic compounds appeared to aggregate from smaller microparticles that fused within the vacuoles (Figures 50-52). The larger phenolic bodies often were appressed closely to the vacuolar membrane (Figure 52). Phenolic compounds also accumulated within the vacuoles of developing and expanded tyloses (Figures 54, 63). All the tyloses that were observed to contain phenolic compounds appeared to accumulate them in the manner mentioned above.
Less frequently, xylem parenchyma cells contained small vacuole-like units with microparticles similar to those which accumulated within the central vacuole (Figure 53). The small vacuoles that originated within the cytoplasm were membrane bound. Details of their origin were not clear. These units in turn occasionally coalesced with the larger central vacuole (Figure 53). In this situation the central vacuole contained a granular substance similar to the tannin bodies observed within the cortical parenchyma cells of the phloem (Figure 55). This electron dense substance within the vacuole contained many holes that apparently resulted from poor infiltration of the embedding medium. These cells may have been undergoing the process of vacuolation and this manner of phenolic accumulation may be incidental to that process.

Ray and vasicentric parenchyma adjacent to vessels containing fungal material did not undergo the accumulation of phenolics described above. The contents of these cells were completely altered so that their cytoplasmic integrity was lost (Figures 27, 28). These cells were therefore considered dead. This situation was observed both within parenchyma cells that had been colonized by hyphae and within cells free of the fungus (Figures 27, 28, 33, 34).

**Tylose formation** Fine structural observations revealed that both ray and vasicentric parenchyma were capable of tylose formation. The vessel-parenchyma pit membranes of these parenchyma cells were directly involved in the formation of tyloses. The pit membrane appeared as the continuation of the middle lamella and the primary cell wall of both the vessel and parenchyma cells (Figures 56, 64, 66). It should be noted, however, that a distinct layering of the membrane was not detected.
The pit membrane exhibited similar continuity and electron density throughout (Figures 64, 66).

A protective layer, around the inside of the parenchyma cell wall, was observed in most xylem parenchyma (Figures 56, 58-62, 64-66). The layer was particularly evident in those parenchyma cells adjacent to vessel elements. In most cases, the protective layer extended completely around the parenchyma cell but was always noticeably thicker near the vessel-parenchyma pit membrane (Figures 46, 47, 56, 58, 59). The layer was so similar to the pit membrane in electron density that frequently the two could not be distinguished (Figures 60, 64, 66). The microfibrillar orientation of the protective layer likewise appeared identical to that of both the vessel-parenchyma and intertracheary pit membranes (Figures 64, 66).

Upon tylose initiation the pit membrane and the protective layer were forced through the vessel parenchyma pit into the vessel lumen by the expanding protoplast (Figures 56, 58, 61). The pit membrane did not rupture during the expansion (Figures 61, 65). The ultimate expansion of the tylose was limited only by the size of the vessel lumen or by its contact with other expanding tyloses (Figures 60-62). During this expansion the protective layer appeared to accumulate additional wall material since the thickness remained relatively constant throughout the expansion process. The primary and secondary cell walls of the xylem parenchyma and the vessel involved in tylose formation remained intact during tylose development (Figures 58-63).

Mature tylose walls were comprised of a normal set of wall layers, i.e., middle lamella, primary wall and secondary wall (Figure 63).
The protective layer appeared analogous to the primary cell wall (Figures 62, 63). During the expansion the pit membrane remained closely affixed to the protective layer and thus could serve as middle lamella between adjoining tyloses or tyloses and the vessel wall (Figures 61, 63, 65). After full tylose expansion the protoplast reverted to synthesizing secondary wall material (Figure 62). This wall material was laid down both within the newly formed protective layer within the vessel and within the existing parenchyma cell. This deposition apparently bridges the existing membranes, presumably rendering them functionless (Figures 61-63, 65). Thus, the parenchyma cell from which the tylose formed contained not only its secondary cell wall but also an additional secondary wall separated by the protective layer (Figure 63).

The young tylose appeared to bud as it pushed through the pit aperture. The bud was densely filled with cytoplasm (Figures 56, 58, 59, 64, 66). As the tylose enlarged the cytoplasm became filled with small vacuoles that appeared bounded by a membrane (Figures 59, 61, 67). The vacuoles enlarged by coalescing with adjacent vacuoles (Figures 60, 67). The parenchyma cell from which the tylose originated likewise underwent vacuolation (Figures 59, 60, 61). Although no quantitative measure was made, I believe that new cytoplasm was not formed during tylose expansion, for the increase in cell volume could be accounted for by the increase in the size of the vacuole.

The structure and number of organelles within developing tyloses were similar to the structure and number of organelles of control xylem parenchyma cells (Figures 45-47, 58, 61). Chloroplasts, frequently not associated with woody tissue, were observed, undoubtedly because
the twigs were only two years old. The organelles of the tylose and the parenchyma cell from which it formed were obviously similar, for the organelles moved from the parenchyma cell through the pit during tylose formation (Figures 58, 59, 61). A layer of cytoplasm remained adjacent to the protective layer throughout vacuolation, so that even after tylose maturity a thin layer of cytoplasm completely surrounded the plasmalemma (Figure 63). The cytoplasm contained ER, ribosomes and numerous dictyosomes (Figures 66-68). Many vesicles were noted and appeared closely associated with both the ER and dictyosomes (Figures 62, 67, 68). In many instances, it seemed that they functioned in either cell wall apposition or vacuolation (Figures 67, 68).
CONCLUSIONS AND DISCUSSION

The progression of host-pathogen responses was quite similar within the selections studied. The characteristic host reaction of the tissue was observed always to be associated with the presence of C. ulmi. A "reactive zone" radiated from the locus of fungal material in all selections, with a greater intensity of reaction within tissue nearest the fungus-infected vessels. Thus, within the reactive zone there is a gradient of host responses to C. ulmi. This situation is suggestive of the host-response system proposed by Kerling (1955).

The reactive zone within U. americana always involved a greater area of xylem tissue. Likewise, more vessels of American elm contained conidia than did vessels of the other selections. This would result in a greater distribution of symptom inducing metabolites and thus explain the larger reactive zone. Workers have shown marked differences in the xylem anatomy among resistant and susceptible varieties (McNabb, et al., 1970). They suggest that the greater number of large contiguous vessels results in greater distribution of the fungus within the susceptible host.

Therefore, I can not agree with Beckman (1966) that a rapid host reaction results in the isolation of the pathogen within the resistant host; close observation suggests instead that the reactions of each selection were quite similar and progressed at the same rates. At least this situation has been established for the first two weeks after inoculation. The only apparent difference in disease development among the selections is that the reactive zone in U. americana always involved a greater area of xylem tissue.
The reaction of elm tissue to *C. ulmi* always proceeded from the vicinity of fungal material and resulted in the reactive gradient mentioned earlier. This was such a consistent feature of disease development that even when the fungus was not evident in sectioned material, the vessel or vessel groups that contained the fungus could often be established. This gradient of tissue reaction was very evident in the three selections studied. The area I have termed the reactive zone was influenced to a certain degree by the inoculation techniques employed. The inoculations initially limited the fungus to a small group of vessels and, because the branch samples were taken in close proximity to the inoculation point, the conidia were limited to vessels that were contiguous with that point. This represents somewhat of an artificial situation when compared to natural inoculation by the elm bark beetle. The beetle, during feeding, may introduce spores into a larger area of the xylem than the pin-prick technique employed in this study. In either case, however, the inoculum is introduced into a limited number of vessels. Therefore, I believe that symptom progression is quite similar and that the reactive zone is typical of either situation.

Examination of cross sections of the inoculated tissue frequently revealed that a single vessel or several vessels contained a large number of spores, undoubtedly resulting from the inoculation with a large spore load. Probably fewer spores are introduced by the elm bark beetle while feeding, merely because the beetle carries a limited number of spores. I suggest that in either circumstance the reactions of the host tissue after inoculation are similar. However, after beetle inoculation of *C. ulmi* the initial establishment period is longer.
The presence of a large number of spores within the vessels was helpful in establishing patterns of fungal growth and movement. Many investigators have had difficulty in observing fungal material within the infected host because the smallness of both the conidia and the hyphal filaments made such observations difficult. The use of the electron microscope answered many questions that have appeared in the literature about fungal growth and movement.

Once the fungus was introduced into the host, colonization commenced. The conidia apparently move quite freely within the vessels into which they have been introduced. Their movement is probably dependent upon the transpiration stream, as has been suggested by other researchers. By conidial germination and the penetration of cell walls or intertracheary pit membranes the fungus grows into adjacent vessels or vessel groups. Many short hyphal strands were seen within xylem tissue, and likewise may serve in the lateral colonization of host tissue. Different stimuli are seemingly responsible for determining whether conidia germinate to form hyphal filaments or bud to form other conidia. However, the conidial form of the fungus presumably would be more effective in colonizing the host. The conidia, by germinating to form hyphae, not only are able to penetrate cell walls and intertracheary pit membranes but also probably have the advantage of a greater mobility within the transpiration stream.

The growth of the fungus through membranes and primary and secondary cell walls suggests that the fungus possesses the enzyme systems necessary for the dissolution of these obstacles. In vitro studies of C. ulmi have demonstrated both cellulase and pectinase activity (Beckman,
1956; Husain and Dimond, 1958). The penetration of the cell walls of elm also may involve the production of lignases. *C. ulmi* may be capable of decomposing lignin even though the production of such enzymes is usually associated with various basidiomycetes. The growth and movement of *C. ulmi* within elm tissue appear to be quite similar to that described for some wood-decay fungi (Cowling, 1965). During the initial stages of colonization *C. ulmi* seemed to exert only a local influence on the cell walls, resulting in penetration and forming a bore hole similar to those formed by many wood-decay fungi. However, later stages of colonization may result in a more extensive breakdown of host cell walls involving the generalized action of enzymes upon the tissue.

The penetration of the fungus through cell walls was irregular. My own observations are that the fungus does not follow the path of least resistance, for it appeared that the penetration depended partly upon the angle of approach to the wall. With a low angle of approach the contact growth was longer and the fungus frequently did not penetrate. With a high angle of approach there may be no contact growth and thus direct penetration. An exception may be when both vessel-parenchyma and intertracheary pit membranes are penetrated. These membranes appear to be penetrated by the fungus more readily than the secondary cell walls, and the penetration here does not appear dependent upon the angle of approach.

The concept that facultative parasites kill the host cells and then live saprophytically on the cell materials is applicable in this situation. The ray and vasicentric parenchyma cells that surround the vessels containing *C. ulmi* completely lose their cytoplasmic integrity, and for
this reason are considered dead. Death may result from the action of particular enzymes or toxic metabolites produced by the fungal parasite. Further examination of the reactive zone has indicated that parenchyma cells not directly associated with the vessels that contain fungi are also altered. These cells, however, do not die but begin to accumulate phenols within their vacuoles. This situation again suggests that the fungus exerts a greater influence upon the cells in close association with the fungus and a lesser influence on those cells some distance from the fungus. Thus, a gradient of response is established.

The growth patterns of the fungus leads to additional speculation about fungus nutrition. Many of the essential nutrients, i.e., amino acids and mineral compounds, are found within the cell sap. Perhaps many of these materials are available from the xylary fluid. Much of the plant carbohydrate, however, is undoubtedly in the form of sugar, starch and cellulose and nitrogen is in the form of protein. To be able to use these materials, the fungus must possess the enzymes which change these into simple, readily assimilated compounds. Such enzymatic activity may be one explanation for the marked decrease in the number of starch grains within infected tissue. The colonization of host tissue may likewise be influenced by the distribution of starch and other usable nutrients. This could also explain the observed colonization of disrupted xylem parenchyma surrounding vessels containing fungal material. Cellulose decomposition is generally associated with later stages of colonization. However, penetration of cell walls suggests that cellulolytic and pectolytic enzymes are secreted at this early stage of colonization. The fungus therefore may also derive a portion of its
nutrition from these complex materials.

The electron density of the intertracheary pit membrane was altered in all species examined. This distinct change might be anticipated because this membrane, consisting of the complex of primary cell wall and middle lamella, remains unprotected by the secondary cell wall, and is left completely exposed to metabolic alteration. The enzymatic activity following infection undoubtedly alters one or more of the components of the membrane. The intertracheary membranes of control material were difficult to characterize because they were similar in electron density to the embedding medium. From what could be observed, however, they too appeared granular.

The alteration of the cell-wall surface within the bordered pit in diseased elm could be attributed to migration of granular material from the pit membrane to the cell wall surface where it accumulates. On the other hand, a portion of the primary cell wall layer may remain affixed to the inner surface of the bordered pit wall during pit formation and it is this portion which in turn is altered by a similar mechanism.

I believe that the overall darkening observed within the reactive zone is caused by the accumulation of oxidized phenolics within both the xylem parenchyma and developing tyloses, and by the discoloration observed in the pit membrane. At the early stages of colonization studied, these were the only discernible areas of discoloration noted using the 1-micron sectioning technique. Furthermore, I believe that if we were to consider the appearance of thicker tissue sections, then the multiplicity of small localized areas of darkening would appear as a large general darkening and give the appearance of discoloration
typically associated with diseased elm tissue.

Several aspects of phenol accumulation should be considered. First, osmium tetroxide produces dark colors with phenols, and for this reason, the darkened areas observed in both the light and electron phases of this study may not be discolored in fresh material. However, preparation of the material in this manner definitely indicates the accumulation of phenolics as the result of infection by C. ulmi.

Apparently two specific processes are involved in the discoloration. First there is the marked increase in phenolic compounds resulting from infection. Secondly, these compounds are oxidized to form the discoloration observed in unfixed diseased elm wood. Because of the preparation used during this study, neither the phenols nor their oxidized products could be clearly distinguished. However, there is some indication of two distinct products.

Frequently xylem parenchyma cells were observed that were almost completely filled with a more granular substance. Infiltration of the embedding medium and sectioning of these cells was extremely difficult; so difficult that often the contents of these cells were pulled out during sectioning, leaving only the xylem parenchyma lumen. Furthermore, the tannin substances within cortical cells of the phloem (Figure 55) were similar in texture and electron density to the granular material viewed within these xylem parenchyma (Figure 45). Therefore, I think that this material could possibly be the oxidized phenols which are visible in fresh material. This suggests that the less granular material seen accumulating within the cell vacuoles represented the unoxidized phenolic compounds.
Neither a quantitative nor qualitative measure of phenol accumulation was made during this study. Therefore, I can only suggest that no visual differences among selections in either the appearance or the amount of accumulated phenol were observed. Further quantitative and qualitative study of this aspect of disease development may provide useful information about other possible resistance mechanisms.

Many investigators have suggested that discoloration was first evidenced in the cell wall. If so, phenolic compounds might diffuse into the cell wall, causing an alteration in electron density. Throughout this study phenolics did not appear to impregnate the cell wall. Furthermore, direct oxidation of the lignin content of the cell wall was not observed. Such diffusion or lignin alteration may occur at a later stage of disease development.

The disruption of the cytoplasm and resultant death of the xylem parenchyma adjacent to vessels containing fungal material again suggests the influence of a fungal-induced gradient upon host tissue. Perhaps toxic metabolites produced by *C. ulmi* cause death of the immediate tissues and result in the process of phenolic accumulation in tissue some distance from the fungal influence.

The control elm material contained such abundant starch grains that frequently they completely filled the cytoplasm of the xylem parenchyma. The grains were bounded by a membrane and occasionally associated with chloroplast-like structures. After inoculation there was a marked decrease in the starch content of the xylem parenchyma. Several days after inoculation few, if any, starch grains were observed. This marked decrease in the number of starch grains coincided with the increase in
phenolic products. Similar correlations have been shown in xylem parenchyma undergoing heartwood formation (Frey-Wyssling and Bosshard, 1959; Hillis, 1968). However, the apparent dissolution of the starch grains noted by Wardrop and Cronshaw (1962) was not observed. Such a dissolution nevertheless could account for the many small vacuoles that contain phenolic compounds.

Electron microscopic observations of tyloses raise speculations about possible mechanisms of tylose formation. A stimulus resulting from vascular invasion by the pathogen undoubtedly initiates tylose budding. Whether tylose formation is initiated solely by a fungal stimulus or by a host-pathogen induced stimulus is yet to be determined. At present the nature of this stimulus can only be speculated. The expansion and growth of the cell wall (protective layer-pit membrane complex) during tylose formation suggest that hormonal activity is directly involved. Experimentation by Ray (1962) indicated that indole acetic acid (IAA) is directly responsible for a portion of the total wall synthesis involved in elongation. He suggested that a certain amount of cell-wall material is incorporated in the existing wall under the influence of IAA, and it is this new material that contributes to the extensibility of the wall. Hence, IAA is able to increase the synthesis of both the pectin and cellulose fraction of the wall. Fungal or fungal-induced IAA synthesis therefore could be responsible for tylose formation. Studies of other wilt diseases (see page 16) suggested possible relationships between IAA and tylose formation (Sequeira and Kelman, 1962; Mace and Solit, 1966).

Tylose formation was never observed within vessels containing C. ulmi.
This could further indicate the involvement of a hormonal stimulus. A hormonal gradient could exist from an area of high concentration in vessels containing fungal material, diffusing to a lesser concentration in adjacent vessels. Tylose formation would thus be inhibited in the area of high hormonal concentration, the lesser concentration of adjacent vessels resulting in tylose stimulation. A similar relationship was formulated by Kerling (1955).

It was also observed that the ray and vasicentric parenchyma adjacent to fungal-containing vessels were apparently killed. Therefore the influence of the fungus, resulting in the death of these cells, may render them incapable of forming tyloses, rather than produce a hormonal concentration that would inhibit tylose formation.

The protective layer-pit membrane complex is highly elastic and conforms to the shape of tyloses that distent it. The elasticity of this complex suggests its similarity to elongating cells of meristematic tissue. This comparison alone, however, does not prove that its wall structure is similar to that of the primary walls in developing meristems. Further comparison of the primary wall-middle lamella complex and the protective layer-pit membrane complex shows a distinct similarity between the two. The similarity in electron density and texture suggests that the microfibrillar structure of the protective layer is randomly oriented. Meyer and Coté (1968), however, found that the microfibrillar organization and electron density of the protective layer of Quercus alba tyloses resembles those of the secondary cell wall.

The developing tylose in elm does not indicate this relationship. The secondary cell wall is distinctly of a lesser density and different
texture than the protective layer, middle lamella and primary wall. This is further substantiated by the identity of the fully developed secondary cell wall of tyloses with that of the secondary cell wall in both the vessel and parenchyma cell with which it is involved. For these reasons I suggest a definite analogy exists between the expanding protective layer-pit membrane complex and the primary cell wall-middle lamella of the xylem tissue. Differences between the cell-wall ontogeny of the tyloses of elm and oak or differences in fixation may account for the observed inconsistency. A system of tylose development and maturation is proposed (Figure 69).

The rupture of the pit membrane, as previously described by Meyer (1967), was not observed. His studies concerned wound-induced tyloses in Quercus alba. This inconsistency may therefore result from either the differences in species or in stimuli.

Beckman (1969) concluded that diurnal changes in the pH of infected tissue resulted in the swelling of walls (loosening of interpolymer bonds in the amorphous cell-wall matrix) in the presence of accumulated CO$_2$ and/or carboxylic acids. The swelling of the perforation plates within infected banana vascular tissue was measurable with the light microscope. Such alteration of either primary or secondary cell walls was not evident within the elm tissue examined. Thus the swelling mechanism reported above may be absent in infected elm tissue, or operating at a greatly reduced rate. Even though I have not viewed the swelling of tissues, the loosening of inter-polymer bonds could be partly responsible for the expansion of the protective layer-pit membrane complex.

The processes involved in tylose wall formation are apparently
similar to the processes of wall formation in other plant cells. In brief summary, the relationship between the elements of the endoplasmic reticulum (ER) and the development of annular thickening in Allium root tips has been suggested (Porter and Machado, 1960; Porter, 1961). Porter (1961) further suggested that the enzyme systems concerned with cell-wall deposition can be synthesized and transported to the cell surfaces by the well-developed ER and the attached polyribosomes. Similar observation of dictyosomes suggest that they may have a secretory function in various types of plant cells (Mollenhauer and Whaley, 1962; Bonneville and Voeller, 1963). Mollenhauer and Whaley (1962) observed that dictyosomes are found in the region of cell-plate formation and that vesicles derived from them aggregate to form the initial stage of the cell plate. These workers also reported that the plate is extended by the fusion of the vesicles at the edges, which indicates that the dictyosomes are contributing both wall material and material to extend the plasmalemma. Furthermore, Moor and Muhlethaler (1963) have shown that the fibrils of the cell wall are produced by special surface areas of the plasmalemma and that the dictyosomes produce only the cell-wall matrix consisting primarily of hemicelluloses.

The numerous dictyosomes and the endoplasmic reticulum found in the expanding tylose undoubtedly contribute in a similar manner to tylose wall apposition. Abundant vesicles appear to originate from both the dictyosomes and the ER. The membrane-bound vesicles possibly contain precursors for the cell-wall matrix, because their fusion with the tylose wall is frequently evident. A system such as this would allow for the expansion of both the cell wall and the plasmalemma.

Microtubules usually found in close association with developing wall
thickenings were not observed. This, however, could readily be due to the fixation technique employed.

Dictyosomes and ER likewise appeared to function in the process of vacuolation of the expanding tyloses. Abundant small vacuoles, bound by a membrane, apparently coalesce to form larger vacuoles. The origin of the small vacuoles can only be hypothesized. However, regions of the ER appear to inflate and form small vacuoles, as previously proposed (Buvat, 1961). Similarly it can be speculated that the small vacuoles are derived from the dictyosome system. This phenomenon was previously reported by Marinos (1963).

In conclusion, this study has provided the first electron microscopic observations of diseased elm tissue, so that many of the internal host responses associated with Dutch elm disease have been clarified. However, there are still many unanswered questions and likewise many new ones. The mechanisms of phenol accumulation, tylose formation, and fungal growth and movement each deserve more intensive study. Future research also should include resistant species so that deviations from the susceptible reaction can be identified. Only then can we begin to fully understand the complexity of this host-pathogen relationship.
LITERATURE CITED


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**APPENDIX A: FIXATION, DEHYDRATION, INFILTRATION AND EMBEDDING**

**Fixation**

Osmium in phosphate buffer:

1% osmium tetroxide in 0.05 m phosphate buffer at pH 7.2; fix one hour at 4° C.

1. Preparation of phosphate buffer (Lillie, 1954):

   0.1 M KH₂PO₄ 13 ml
   0.1 M Na₂HPO₄ 37 ml

   Adjust to pH 7.2 - 7.4 if necessary.

2. Dilute phosphate buffer with 2% osmium tetroxide 1:1.

**Dehydration and Infiltration**

Epon and Araldite-Epon specimens were dehydrated in the following manner at room temperature. All specimens were rotated on a slow rotating mixer throughout dehydration and infiltration.

**Dehydration:**

1. 5 minutes each in 2 changes, 50 percent ethanol
2. 5 minutes each in 70, 95 percent ethanol
3. 3 changes of 5 minutes each in absolute ethanol
4. 3 changes of 5 minutes each in propylene oxide

**Infiltration:**

1. 15 minutes in a mixture of 1 part Epon (Araldite-Epon):
   
   3 parts propylene oxide

2. 60 minutes in a mixture of 1 part Epon (Araldite-Epon):
1 part propylene oxide

3. 2 hours in a mixture of 3 parts Epon (Araldite-Epon):
   1 part propylene oxide

4. 12-18 hours pure Epon (Araldite-Epon)

5. Specimens were embedded in shallow boats made of aluminum foil.

6. Stepwise polymerization was accomplished at 35°C for 12 hours, 45°C for 12 hours and 60°C for 1 week.

Fixation, dehydration and infiltration following the above method proved very satisfactory. The major tissue damage resulted from mechanical injury during the sectioning prior to fixation. Other fixation procedures would undoubtedly have provided other information that could be useful to future investigations.

**Embedding**

Specimens were embedded in Epon 812 (Luft, 1961); and Araldite-Epon (Mollenhauer, 1964; Anderson and Ellis, 1965).

**Epon 812:**

1. Mixture A
   62 ml of Epon 812
   100 ml of dodecenyl succinic anhydride (DDSA)

2. Mixture B
   100 ml of Epon 812
   89 ml of nadic methyl anhydride (NMA)

3. Add mixture A to B in 3:2 ratio

4. Add 0.2 ml of DMP-30 (catalyst) per 10 ml of Epon mixture.
Araldite-Epon:

1. Araldite 502 10 ml
2. Epon 812 13 ml
3. DDSA 30 ml
4. DMP-30 2 ml
APPENDIX B: KEY TO LABELLING

C  conidia  
CH  chromatin-like body  
CL  chloroplast-like body  
D  dictyosome  
ER  endoplasmic reticulum  
HP  hyphal pore  
IM  intertracheary membrane  
M  mitochondria  
ML  middle lamella  
N  nucleus  
OG  osmiophilic granule  
P  phenolic compounds  
PD  plasmodesmata  
PL  protective layer  
PM  vessel-parenchyma membrane  
PW  primary wall  
S  starch  
SW  secondary cell wall  
T  tylose  
TB  tannin body  
V  vacuole  
VL  vessel lumen  
VS  vesicle
APPENDIX C: FIGURES
Figure 8. Cross section of *U. americana*, from control sample. Note large, contiguous vessels, and abundant starch grains within xylem parenchyma. Line scale represents 40 microns

Figure 9. Cross section of *U. carpinifolia 'Christine Buisman*', from control sample. Note small but contiguous vessels and abundant starch grains within xylem parenchyma. Line scale represents 40 microns

Figure 10. Cross section of *U. pumila var. Chinkota*, from control sample. Note large but isolated vessels and abundant starch grains within xylem parenchyma. Line scale represents 40 microns
Figure 11. *U. americana*, one day after inoculation. Note tyloses within several vessels and phenolic compounds within xylem parenchyma. Line scale represents 40 microns.

Figure 12. *U. carpinifolia* 'Christine Buisman', one day after inoculation. Note conidia within a single vessel, tyloses in adjacent vessel group, discoloration of intertracheary pit membranes (small arrows) and phenolic compounds within xylem parenchyma. Line scale represents 40 microns.

Figure 13. *U. pumila* var. Chinkota, one day after inoculation. Note conidia about the periphery of large vessel, discoloration within numerous parenchyma, discoloration of intertracheary pit membranes (small arrows) and tyloses in several vessels. Line scale represents 40 microns.
Figure 14. *U. americana*, three days after inoculation. Note larger area of tylose formation than in Figures 15, 16, also increased phenolic accumulation within parenchyma, conidia and discolored intertracheary pit membranes (small arrows). Line scale represents 40 microns.

Figure 15. *U. carpinifolia* 'Christine Buisman', three days after inoculation. Note increased number of parenchyma containing phenolics, discoloration of numerous intertracheary pit membranes (small arrows) and conidia completely filling vessel lumen. Line scale represents 40 microns.

Figure 16. *U. pumila* var. Chinkota, three days after inoculation. Note phenolic accumulation within tylosis and discoloration of intertracheary pit membranes (small arrows). Line scale represents 40 microns.
Figure 17. *U. americana*, five days after inoculation. Note abundant tyloses within most vessels in the field of view, thickening of the tylose wall (small arrows) and discoloration of the intertracheary pit membranes. Line scale represents 40 microns.

Figure 18. *U. carpinifolia* 'Christine Buisman', five days after inoculation. Note the accumulation of phenolic compounds within numerous ray parenchyma cells. Line scale represents 40 microns.

Figure 19. *U. pumila* var. Chinkota, five days after inoculation. Note phenolics within tyloses and many parenchyma cells. Reactive zone limited to several vessel groups unlike Figure 17. Line scale represents 40 microns.
Figure 20. *U. americana*, nine days after inoculation. Note reactive zone spreading beyond field of view even in lower magnification micrograph. Line scale represents 40 microns.

Figure 21. *U. carpinifolia* 'Christine Buisman', nine days after inoculation. Note reactive zone limited to several vessel groups and a smaller area than in Figure 20. Line scale represents 40 microns.

Figure 22. *U. pumila* var. Chinkota, nine days after inoculation. Note limited reactive zone. Line scale represents 40 microns.
Figure 23. *U. americana*, twelve days after inoculation. Note conidia (small arrows) in numerous vessels and tylose formation extending beyond field of view (extensive reactive zone). Line scale represents 40 microns.

Figure 24. *U. carpinifolia* 'Christine Buisman', twelve days after inoculation. Note vessel containing conidia and abundant tyloses. Line scale represents 40 microns.

Figure 25. *U. pumila* var. Chinkota, twelve days after inoculation. Note limited reactive zone, abundant phenolic accumulation and thickening of tylose wall (small arrows). Line scale represents 40 microns.
Figure 26. Light microscopic cross section of area associated to section in Figure 27. Line scale represents 40 microns

Figure 27. Cross section of *U. americana*, three days after inoculation showing disruption of cytoplasm within vasicentric parenchyma adjacent to vessel containing conidia. Line scale represents 2 microns
Figure 28. Cross section of *U. pumila* var. Chinkota, three days after inoculation. Note disruption of cytoplasm within vasicentric parenchyma adjacent to vessel A that contains conidia. Cytoplasm within parenchyma surrounding vessel B is unaltered. Line scale represents 2 microns.
Figure 29. Light microscopic cross section of area associated to section in Figure 30. Line scale represents 40 microns

Figure 30. *U. carpinifolia* 'Christine Buisman', five days after inoculation showing hyphal penetration of intertracheary pit membrane (A) and vessel-parenchyma pit membrane (B). Note accumulation of phenolic compounds within vacuole of ray parenchyma. Line scale represents 2 microns
Figure 31. Enlargement of Figure 30 showing penetration of intertracheary membrane. Note ER, mitochondria and simple pore within hyphal filament. Line scale represents 1 micron
Figure 32. Conidium with internal wall structure possibly indicating the budding mechanism found in *C. ulmi*. Line scale represents 1 micron.

Figure 33. *U. pumila* var. Chinkota, nine days after inoculation showing fungal penetration of disrupted vasicentric parenchyma cell through the vessel parenchyma pit membrane. Note simple pore and nucleus in *C. ulmi*. Line scale represents 1 micron.
Figure 34. *U. pumila* var. Chinkota, five days after inoculation showing penetration of vessel-parenchyma pit membrane by hypha of *G. ulmi*. Note disruption of cytoplasm within vasicentric parenchyma, the presence of fungus (A) within the cell and osmiophilic granules within hypha. Line scale represents 1 micron.
Figure 35. Light microscopic cross section of area associated to section in Figure 36. Line scale represents 40 microns

Figure 36. *U. carpinifolia* 'Christine Buisman', nine days after inoculation showing both penetration of the cell wall and intertracheary pit membrane by hypha of *C. ulmi*. Note growth across vessel lumen to opposite cell wall. Line scale represents 1 micron
Figure 37. *U. carpinifolia* 'Christine Buisman', five days after inoculation showing the penetration of hypha of *C. ulmi* through two intertracheary pit membranes. Note the conformity of the hypha of *C. ulmi* to the walls of the pit apertures, also vacuolation and numerous mitochondria within hyphal strand. Line scale represents 2 microns.
Figure 38. *U. carpinifolia* 'Christine Buisman', five days after inoculation showing cell wall penetration. Note expansion of hypha of *C. ulmi* after penetration and zone of greater electron density (arrows) on either side of fungus. Line scale represents 1 micron.

Figure 39. *U. carpinifolia* 'Christine Buisman', five days after inoculation showing penetration of the secondary cell wall, primary cell wall and middle lamella by hypha of *C. ulmi*. Note in median section area of dissolution preceding fungus (arrow), also note altered pit membranes. Line scale represents 1 micron.
Figure 40. *U. carpinifolia* 'Christine Buisman', five days after inoculation showing penetration of secondary cell wall by hypha of *C. ulmi*. Note zone of greater electron density on either side of penetration peg (arrows). Line scale represents 0.5 micron.
Figure 41. *U. pumila* var. Chinkota, nine days after inoculation showing penetration of pit membrane and the direct penetration of secondary cell wall on opposite side of aperture by hypha of *C. ulmi*. Line scale represents 1 micron.
Figure 42. *U. carpinifolia* 'Christine Buisman', from control sample. Note abundant starch grains within xylem parenchyma, also the intertracheary pit membranes are of density similar to the embedding medium. Line scale represents 2 microns.
Figure 43. *U. carpinifolia* 'Christine Buisman', from control sample. Large group of adjoining vessels, note unaltered membranes of density similar to the embedding medium. Line scale represents 2 microns.

Figure 44. *U. carpinifolia* 'Christine Buisman', from control sample. Ray parenchyma containing abundant starch grains within chloroplast-like bodies. Line scale represents 2 microns.
Figure 45. *U. pumila* var. Chinkota, from control sample. Abundant starch grains within xylem parenchyma. Note osmophilic granules and natural occurring tannin body similar to those in Figure 55. Line scale represents 2 microns.
Figure 46. *U. americana*, from control sample. Abundant starch grains frequently associated with chloroplast-like bodies. Note protective layer. Line scale represents 2 microns.
Figure 47. *U. americana*, from control sample. Abundant starch grains associated with chloroplast-like bodies. Note protective layer that is evident on side of parenchyma adjacent to vessel. Line scale represents 2 microns.
Figure 48. Light microscopic cross section of area associated to section in Figure 49. Line scale represents 40 microns

Figure 49. *U. carpinifolia* 'Christine Buisman', three days after inoculation showing the alteration in electron density of the intertracheary pit membranes and the superficial discoloration of the cell wall within the double bordered pit. Line scale represents 2 microns
Figure 50. *U. americana*, one day after inoculation showing micro-particles (phenolics) aggregating within the vacuoles. Note parallel ER, mitochondria and osmiophilic granules. Line scale represents 1 micron

Figure 51. *U. americana*, three days after inoculation showing later stage of phenolic accumulation within many small vacuoles. Line scale represents 1 micron
Figure 52. *U. pumila* var. Chinkota, one day after inoculation showing the aggregation of microparticles (phenolics) about the vacuolar membrane within a ray parenchyma cell. Note this cell still contains starch grains. Line scale represents 1 micron.
Figure 53. *U. americana*, five days after inoculation showing the coalescence of small vacuoles with the central vacuole. Note the difference in texture between the tannin-like substance within the vacuole and the phenolics within the vacuoles of Figures 50-52. Line scale represents 1 micron.
Figure 54. *U. carpinifolia 'Christine Buisman',* five days after inoculation showing the aggregation of phenolic materials within the vacuole of a tylosis. Note altered pit membranes. Line scale represents 2 microns.
Figure 55. *U. americana*, from control sample. Note large tannin accumulation within cortical parenchyma cells of the phloem. This material is similar in density and texture to Figures 45, 53. Line scale represents 1 micron.
Figure 56. *U. carpinifolia* 'Christine Buisman', one day after inoculation showing tylose initiation. Note the cytoplasmic bud appears to force both the protective layer and pit membrane into vessel lumen. Line scale represents 1 micron.
Figure 57. Light microscopic cross section of area associated to section in Figure 58. Line scale represents 40 microns

Figure 58. *U. americana*, three days after inoculation showing an expanding tylose. Line scale represents 1 micron
Figure 59. *U. carpinifolia* 'Christine Buisman', three days after inoculation showing an expanding tylose. Note vacuolation within expanding cytoplasm. Line scale represents 1 micron.
Figure 60. *U. pumila* var. Chinkota, five days after inoculation showing large vacuolar area. Note that the tylose has extended and contacted another expanding tylose. Line scale represents 1 micron.
Figure 61. *U. carpinifolia* 'Christine Buisman', five days after inoculation showing a fully expanded tylose. Note the limited vacuolation probably due to its expansion into a small vessel. Line scale represents 2 microns.
Figure 62. *U. pumila* var. Chinkota, nine days after inoculation showing two fully expanded tyloses. Note the numerous vesicles associated with the protective layer. Line scale represents 2 microns
Figure 63. *U. americana*, twelve days after inoculation showing mature tyloses. Note intertylosic pitting and the complement of cell wall layers, i.e., middle lamella, primary cell wall and secondary cell wall. Line scale represents 2 microns.
Figure 64. *U. carpinifolia* 'Christine Buisman', one day after inoculation showing cytoplasmic bud. Note abundant ER and ribosomes. Line scale represents 1 micron.

Figure 65. Enlargement of Figure 61. Note the expanded pit membrane and protective layer. Line scale represents 1 micron.
Figure 66. Enlargement of Figure 64. Note the similarity of the protective layer and pit membrane in density and texture. Line scale represents 1 micron.
Figure 67. Enlargement of Figure 59. Note irregularity of the expanding protective layer (arrows). Also note dictyosomes, vesicles (small arrows), vacuoles and ER. Line scale represents 1 micron

Figure 68. Enlargement of Figure 58. Note vesicles (small arrows) associated with dictyosomes, small vacuoles and ribosomes. Line scale represents 1 micron
Figure 69. A proposed system for tylose development and maturation