Colonization of Resistant and Susceptible Oaks by *Ceratocystis fagacearum*

W. R. Jacobi and W. L. MacDonald

Graduate research assistant and associate professor of plant pathology, respectively, Division of Plant Sciences, West Virginia University, Morgantown 26506. Present address of senior author: Department of Plant Pathology, North Carolina State University, Raleigh 27650.
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ABSTRACT

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Colonization of northern red oak, *Quercus rubra,* chestnut oak, *Q. prinus,* and white oak, *Q. alba,* by *Ceratocystis fagacearum* from the time of inoculation to symptom expression was investigated by light microscopy. Two- to 3-yr-old branches from stump sprouts of each species were inoculated and stem sections were collected above and below the inoculation point before and after symptoms appeared. Symptoms were expressed by day 28 in red oaks and chestnut oaks and by day 53 in some white oaks. The pathogen was restricted laterally and longitudinally in vessels of white oak. The quantity of hyphae and conidia observed in white oak tissue was relatively constant by the 14th day after inoculation. In red oak, observable hyphae and conidia, initially observed by day 7, increased with time, spreading laterally and longitudinally into many small vessels and tracheids. Tyloses, gums, bubble-like structures, and darkly stained parenchyma cells were observed in all species in response to invasion. The ratio of tyloses to observable fungus was greater in white oaks than in red or chestnut oaks. A zone of darkly stained paratracheal parenchyma cells formed in white and chestnut oaks; in red oak this zone was more diffuse and involved both paratracheal and ray parenchyma. These differences in host responses may be related to the differential rate of colonization in red and white oaks.

Additional key words: oak wilt.

Ceratocystis fagacearum (Bretz) Hunt invades the vascular systems of both susceptible red oaks (Erythrobalanus) and resistant white oaks (Leucobalanus). In red oaks colonization and death is common. Infection is less predictable in the white oaks; death may not occur for several years, or there even may be a recovery.

The host-parasite relationships of oak wilt in resistant and susceptible hosts have not been adequately explained. The problems of working with large trees and the seeming paucity of pathogen propagules in infected tissues prior to symptom expression have limited the study of this aspect of the disease (4,12,13,15–17). Therefore, most cytological studies involving resistant and susceptible oaks have dealt primarily with advanced stages of the disease. As a result, the incidence, form, location of hyphae and conidia in vessels, and related early host responses from inoculation to wilt have not been adequately observed.

The objective of this study was, therefore, to compare host responses, host-pathogen interactions, and fungal colonization of xylary tissue in resistant white and chestnut oaks and susceptible red oaks before and after symptom expression.

MATERIALS AND METHODS

Two- to 5-yr-old stump sprouts of chestnut oak (*Quercus prinus* L.), white oak (*Q. alba* L.) and northern red oak (*Q. rubra* L.), located in the West Virginia University Forest near Morgantown, were used in this study. Two- to 3-yr-old branches were inoculated on 2 July 1974. One milliliter of an inoculum suspension containing approximately 750,000 conidia per milliliter was placed in a plastic reservoir (BEEM embedding capsule) attached with grafting wax. A wound was then made with a 1-mm-diameter drill through the base of the inoculum-filled capsule into the branch tissue (Fig. 1). Water-inoculated controls were treated in the same manner. Five inoculated (replicates) and two control branches were collected from each species 1, 3, 14, 28, 47, 53, and 76 days after inoculation. Most observations were made from cross- and longitudinal sections at point two, 5–7 cm above the inoculation point (Fig. 1). Additional sections were made at point three, 15 cm above, and at point one, 10 cm below the inoculation point when the fungus was observed at point two (Fig. 1).

Stem pieces for isolation of *C. fagacearum* were surface sterilized either by flaming in EtOH or by immersion in a 0.5% sodium hypochlorite solution for 3 min. Four sections of these stems were then placed on a glucose-phenylalanine agar medium for culture (1). Stem pieces for culture were obtained from the three sampling points (Fig. 1).

Material for sectioning was fixed in 10% acrolein, dehydrated in a series of ethylene monoethyl ether, 100% ethanol, *n*-propanol and *n*-butanol, and then embedded in glycol methacrylate [purified glycol methacrylate, 75 ml; 2,2′-azobis(2-methylpropionitrile), 0.02 g, and polyethylene glycol 400, 25 ml] as described by Feder and O'Brien (3). Sections 6–12 μm thick were cut on a rotary microtome with a steel knife. Every sixth-to-eighth section was saved and stained with buffered 0.05% aqueous toluidine blue O dye and mounted with Karo corn sugar syrup. Both bright-field and phase-contrast optics were used to view sections.

The quantification of fungal colonization was made by examining longitudinal sections, whereas the degree of vessel plugging was measured by observing cross sections of vessels $>$ 10 μm in diameter. When observations were made of vessels containing fungus (primarily hyphae), the vessels were grouped and data were recorded by large (>10 μm) and the small diameter(<10 μm) classes. This arbitrary division was used to separate the large vessels with associated parenchyma from the small vessels and tracheids without associated parenchyma.

The distribution and quantification of the fungus at point 2 was originally recorded as the average number of vessels containing fungus in 30–40 sections from each of five branches. Vessel plugging was scored as the average percentage of vessels occluded at point 2 in sections from each of five branches. Because of wide swings in these values, a log transformation of the data was performed. To accommodate the zero values, one was added to each value before taking the logarithm; ie, $y' = \log(y + 1)$. Predictive polynomial models up to the sixth degree in time were fitted to the data. In these models the log transformation of the number of vessels containing fungus per histological section or the amount of vessel plugging were the dependent variables, and time was the independent variable.

Predictive curves using the models which were appropriate for the various situations are shown in the results section (Figs. 2,3). Model selection was by significance pattern of terms; ie, the degree of the model was the highest-order significant term at the $P = 0.01$

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level. The F value for the regression for the overall model was highly significant for each treatment. The significance pattern for the various polynomial terms varied from one treatment to the next.

RESULTS

Isolation and symptom expression. Isolation of the fungus from stem sections was not a useful test for detecting the presence of *C. fagacearum* since hyphae were seen 3 days after inoculation in histological sections, but the pathogen could not be isolated until 28 days after inoculation. Isolation was, however, accomplished 20 days before symptoms were observed in red and white oaks. The fungus was not isolated from chestnut oak until symptoms were visible. Most red and chestnut oaks expressed symptoms in 28–47 days, but 53 days passed before symptoms appeared in white oaks. Symptom expression included wilting, leaf chlorosis, necrosis, and rapid defoliation.

Fungal distribution in the three oak species. Few hyphae or conidia were observed in any oak stem until after wilting. When hyphae were found in vessels and tracheids of xylem tissue, their diameters varied from 0.6 μm to 4.5 μm (Figs. 4A,B). The smaller diameter hyphae were more common in early collections and in newly colonized vessels. In large vessels, the hyphae branched frequently in lumens and also grew along vessel walls. Less hyphal branching was noted in smaller vessels and tracheids. Hyphae and spores clustered at vessel perforation plates where parenchyma cells occasionally were penetrated (Fig. 4E).

Initial colonization, as noted by observable hyphae and spores, was slower in red than in white or chestnut oak (Fig. 2). Eventually, however, both vertical and lateral distribution of the hyphae became more extensive in red oak than in the other two species. The fungus was observed first on day 7 in each of the three oak species. In red oak several small-diameter vessels were invaded by day 14 and in subsequent collections there was a striking increase in the number of small and large vessels that were invaded (Figs. 2A,B). In contrast, very few hyphae were found after the initial colonization in white oak (Figs. 2C,D). The hyphae generally were small in diameter and were usually in large-diameter vessels. The invasion of small vessels did not progress beyond the level reached by day 14

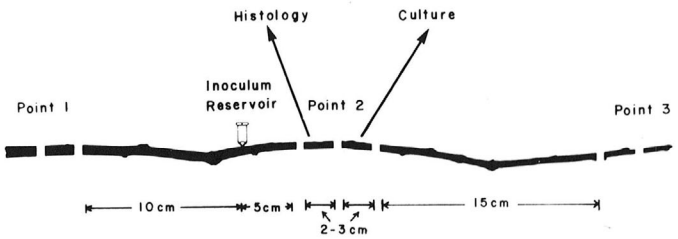

Fig. 1. Sampling of *Ceratocystis fagacearum*-infected oak stump shoots. Positions of sample points on each branch. Point 1 is at the basipetal end and point 3 is at the acropetal end of the branch.

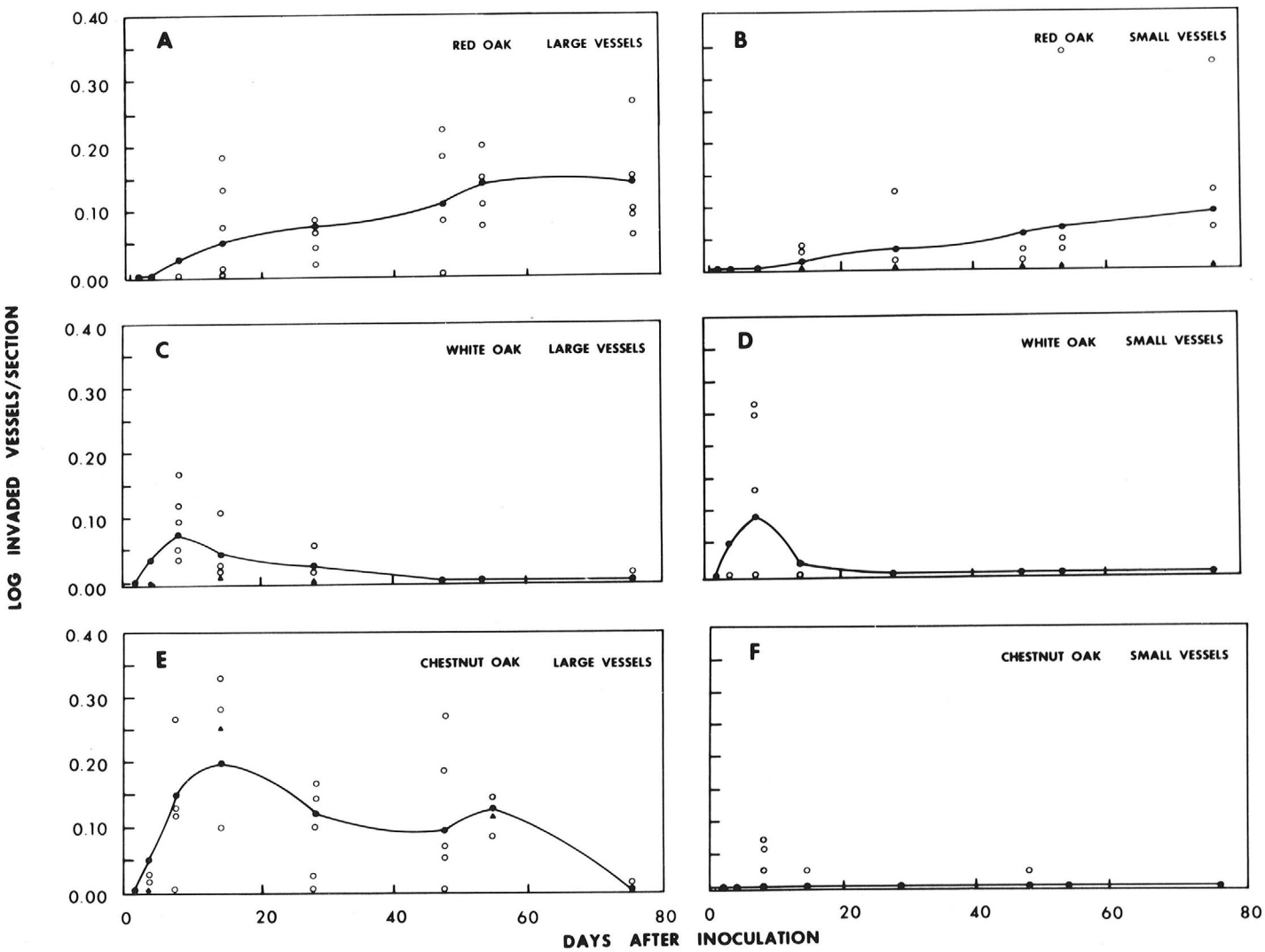

Fig. 2. *Ceratocystis fagacearum* infection of oak shoots. The amount of colonization over time in red, white, and chestnut oak. This is indicated by the log of the average number of invaded vessels per observed histological section, fitted to the appropriate polynomial. Large vessels are > 10 μm and the small vessels are < 10 μm. Legend: o, observed value; ▲, more than one observed value; ●, predicted value; some predicted values overlap observed values.

(Figs. 2C,D). The most fungal material was observed in chestnut oak. Colonization of chestnut oak was common in large vessels with essentially no lateral movement into small vessels (Figs. 2E,F). An exception to this general trend was seen on day 3 with the colonization of a few small vessels (Fig. 4H). Observations of chestnut and red oaks at sample points one and three showed that at 28 and 47 days the amount of vessel plugging and fungal spread either equaled or surpassed the amount at point two. Hyphae also were observed at points one and three in white oak but in lesser amounts than at point two. In the severely wilted stems of red, chestnut, and white oaks on day 76 large occluded vessels contained only pieces of hyphae or pockets of massed hyphae. At this time in red oak, the fungus had spread laterally to many adjacent small vessels (Figs. 2A,B).

Sporulation and fungal growth. Conidia were not commonly observed, but when present were found in the earliest collections. Conidial sizes varied with dimensions ranging from 5.2 μm × 1.0 μm to 7.8 μm × 2.6 μm (Fig. 4C). The diameter of the smaller conidia was less (1.0 – 1.5 μm) than those produced in vitro.

Conidiophores were rarely observed in any of the sampled branches. One conidiophore, possibly small enough to produce the smaller conidia, was observed in a chestnut oak vessel on day 28 (Fig. 4F). Hyphal projections, possibly conidiophores, were observed protruding from pits, especially in chestnut oak on day 7 (Fig. 4G).

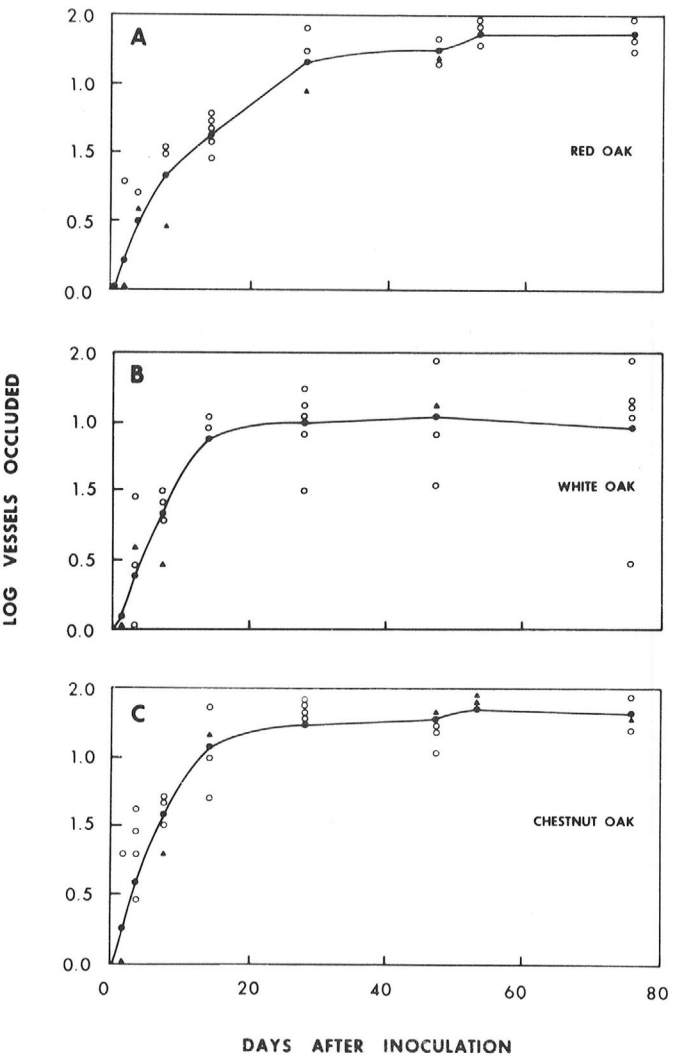

Fig. 3. *Ceratocystis fagacearum* infection of oak shoots. Amount of vascular occlusion in large vessels of red, white, and chestnut oak as depicted by the log of the percent of vessels occluded, fitted to the appropriate polynomial. Legend: o, observed value; ▲, more than one observed value; ●, predicted value.

Growth of the fungus from vessel to vessel occurred by penetration of bordered pits (Fig. 4D). Pit penetration was rarely observed in white oak. Although hyphae were commonly seen passing through vessel walls, it could not be seen clearly whether or not this was the result of direct wall penetration (Fig. 4E).

Host responses to colonization. Darkly stained cells, tyloses, gums, and bubble-like structures formed in the infected areas in all hosts. A sharply defined area of darkly stained parenchyma cells surrounded both colonized and tylosed vessels in white oaks and some chestnut oaks (Fig. 5A). This reaction to the pathogen showed most clearly in later collections, but was observed soon after inoculation. Red oak never formed the distinct discolored zone in the colonized area, but rather showed a darkening of many scattered cells, especially of the ray parenchyma (Fig. 5B).

Tyloses formed readily within 3 days of inoculation in large vessels of all species (Figs. 3,5D,E). In the 14- and 28-day collections, in any one section, 40% or more of the vessels were occluded with tyloses in red and chestnut oaks. Tyloses were not observed in small vessels and tracheids at any time after inoculation. In most collections, chestnut and red oaks were similar in the degree of vascular occlusion (Fig. 3). Even though white oak expressed fewest symptoms, tylosis at point 2 was comparable to that of red and chestnut oak (Fig. 3). Severe wilting occurred in all species when about 50–60% of the vessels in a cross section were occluded. Fewer tyloses were generally found at points 1 and 3 than at point 2 in all three species. Vessels containing the fungus were clear of tyloses or contained very small ones while adjacent vessels were completely occluded with tyloses formed in a matrix of gum (Fig. 5E). Only a very few wound-induced tyloses were seen in water-inoculated controls at point 2.

Gums formed in large vessels and in nearby ray and vasicentric parenchyma cells but were lacking in the small vessels and tracheids (Fig. 5C). Gum deposition in vessels began in all oak species between day 7 and 14. No differences in gum formation were found among the species.

Thin-walled bubble-like structures were observed as early as day 3 in all host vessels either containing or near those containing hyphae (Fig. 5F). Occasionally the bubble-like structures coalesced forming a plugging material. Bubble-like structures were never observed in control stems, and there was no apparent relationship between species of oak and quantity of bubble-like structures.

DISCUSSION

The mechanisms responsible for the apparent restriction or slowing of wilt in white oaks have been hard to demonstrate. Beckman (2) theorized that most plants can localize vascular infections by a process of screening out motile cells or spores at perforation plates with gels and tyloses which seal off the infected area. In susceptible plants, the pathogen overcomes these reactions. This general process of localization of the pathogen appeared to be the case in our study.

The differences in colonization among the three oak species indicate where and how resistance or localization might occur. Lateral and longitudinal restrictions (ie, tyloses, gums, anatomical restrictions, and cellular responses) in white oak may keep the extent of growth of the pathogen well below that in red oak. In white oak, *C. fagacearum* does not appear to be able to move laterally into uncolonized vessels or to be able to bypass blockages caused by gums, tyloses, and plugged perforation plates. Such restrictions may limit or slow the spread within a section of infected xylem, thus protecting portions of a branch or tree.

In contrast, the striking lateral and longitudinal growth of the fungus in red oaks allows for more complete colonization of the entire tree. Unrestricted lateral growth allows the pathogen to bypass plugged vessels. The invasion of small vessels and tracheids could further enhance distribution since small vessels and tracheids lack associated parenchyma cells and therefore the abililty to produce restricting tyloses and/or gums. Longitudinal restriction did not appear to be limited in red oak since generally more fungus was observed at collection points 1 and 3 than at point 2. The slower rate of initial colonization in red than in white and chestnut oaks

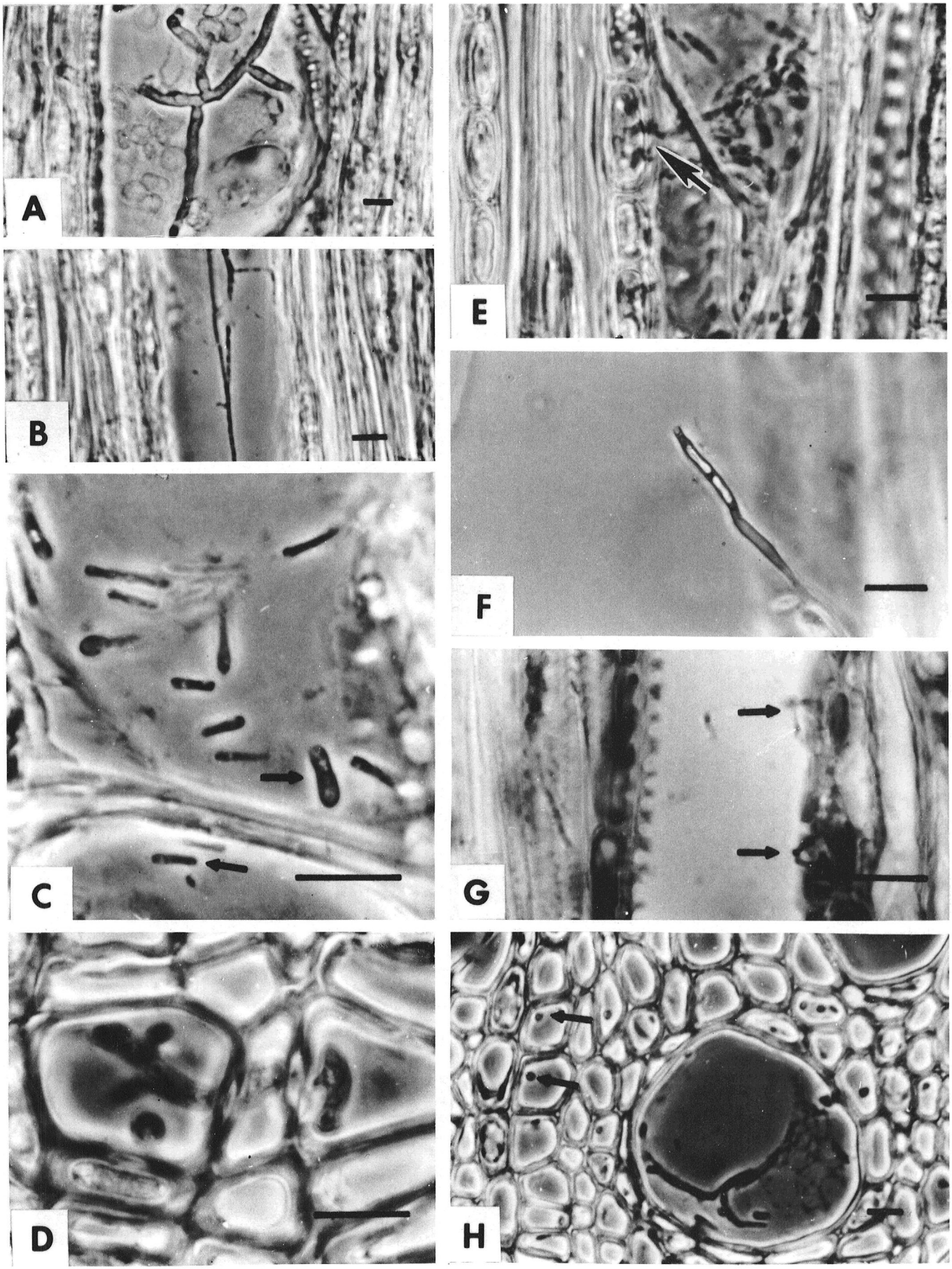

Fig. 4. *Ceratocystis fagacearum* infection in oaks. The appearance of the fungus in colonized tissue. **A,** Branching of large-diameter hyphae in red oak on day 47. **B,** Small diameter hyphae in chestnut oak on day 7. **C,** Small and large conidia (arrows) at a perforation plate in chestnut oak on day 7. **D,** Hyphal penetration of bordered pits in chestnut oak on day 14. **E,** Fungus clustering a perforation plates and penetration of parenchyma cells (arrow) in chestnut oak on day 7. **F,** A small conidiophore in chestnut oak vessel on day 28. **G,** Hyphal projections (arrows) from bordered pits in chestnut oak on day 7. **H,** Hyphae in small vessels and tracheids (arrows) of chestnut oak on day 3. Line scales represent 10 μm except for figure G in which the scale represents 50 μm.

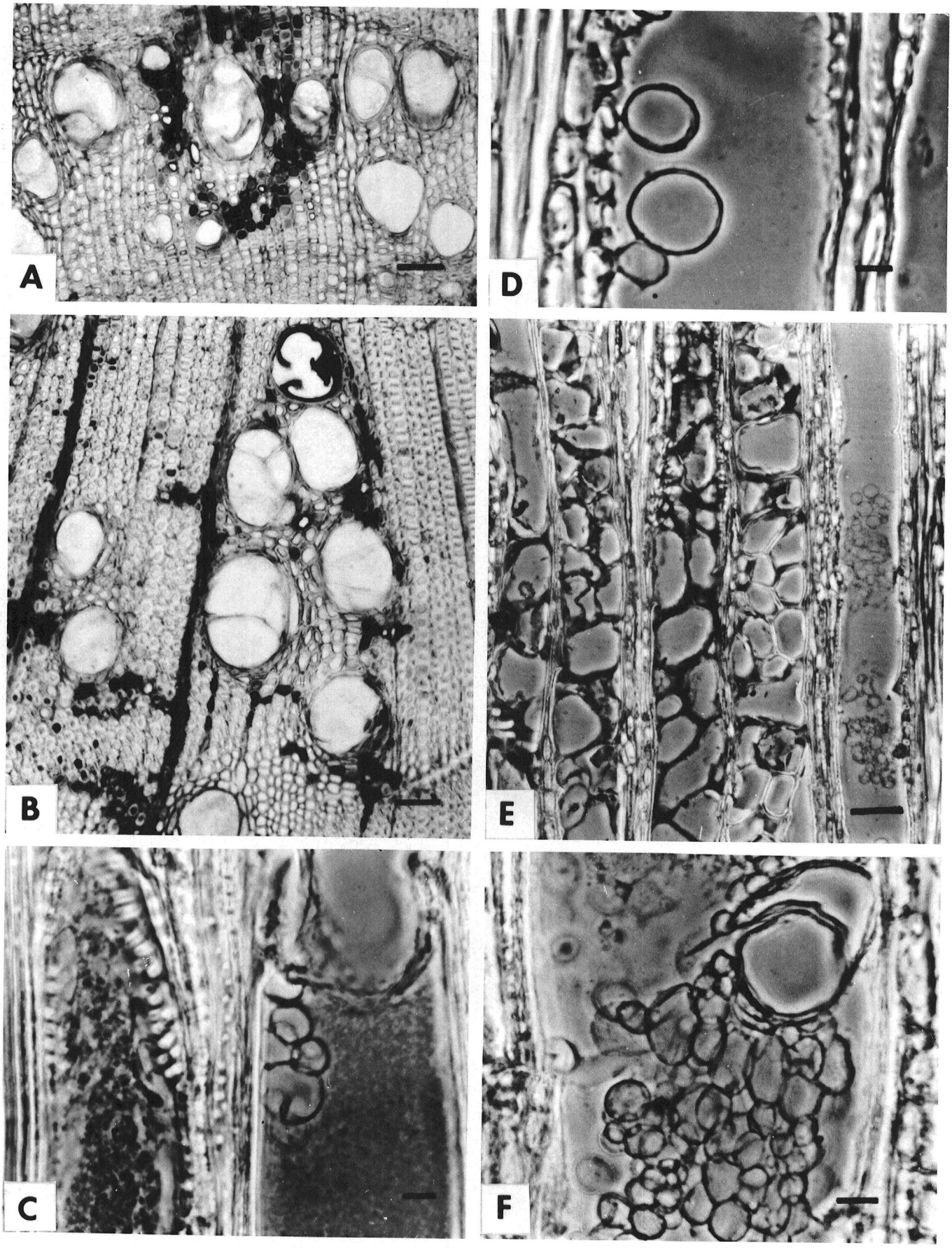

Fig. 5. *Ceratocystis fagacearum* infection of oak shoots. Host reaction to colonization. **A,** A distinct zone of dark-staining parenchyma cells in a cross section of white oak on day 47. **B,** Staining of ray and scattered parenchyma cells in red oak on day 76. **C,** Gums of different consistencies in vessels of red oak on day 28. **D,** Young tyloses forming from vasicentric parenchyma cells in chestnut oak on day 7. **E,** Vessels occluded with tyloses in matrix of gum and colonized vessel with bubble-like structures in red oak, day 47. **F,** Bubble-like structures and a tylose in chestnut oak on day 14. Scale bars for figures A, B, and E represent 50 μm, for figures C, D, and F they represent 10 μm.

was unexpected. A delay in the initial rate of fungal growth in red oak may induce fewer early host responses and ultimately allow for greater colonization.

The general absence of hyphae and conidia in all hosts prior to wilting also has been noted by other workers (4,15–17). The restriction of lateral growth and our inability to readily observe the fungus in vessels prior to wilt may indicate that the host, whether resistant or susceptible, in some way limits hyphal growth until wilting occurs. Failure to reisolate the fungus by culture during the early stages of colonization also may be related to the limited pathogen growth prior to wilting.

Variation in hyphal and conidial size and shape seen here and also reported by Wilson (16) may relate to colonization. Large-diameter hyphae usually were seen in extensively colonized areas of red oak whereas they were lacking in white oak. If hyphal size relates to the nutritional condition of the fungus then it would seem that *C. fagacearum* is able to establish a better nutritional relationship with red oak. This in turn would enhance colonization. In white oak the fungus may be restricted to the nutritional base of the xylem sap. This source of nutrition might result in the production of small hyphae and conidia.

Conidia (1.0 μm in diameter) smaller than those originally introduced and small conidiophores protruding from pits also have been reported in infected oaks (16). The small conidia, similar in size to those produced on water agar, may be associated with poor nutrition. The production of a relatively few atypical conidia during early stages of colonization may be the key to the rapid distribution of the fungus and the reason why others have failed to observe conidia by light microscopy (4,6,12,15,16).

Tylosis, vascular discoloration, gummosis, and bubble-like structure formation were host responses typical of all species. However, the magnitude and type of response differed among them. The distinct or darkly colored zone surrounding colonized white oak xylem elements was striking. The darkly staining compounds may be fungitoxic and prevent the fungus from establishing a nutritional relationship. Such a dramatic response appears to be absent in red oak in which the discoloration was diffuse. The accumulation and/or oxidation of host phenols probably accounts for the discoloration. These compounds appear similar to the phenolics described by Gagnon (5) for *C. ulmi*-infected elm xylem. The discoloration and gummosis are presumably the same as previously reported (9,12,15) and the dark substances in xylem parenchyma like those shown by Sachs et al (13).

Deposition of gums and bubble-like structures in vessels occurred soon after inoculation and near or in colonized vessels. Previously, gums have been observed only in the late stages of wilt (14). The gums and coalesced bubble-like structures may restrict further sap or conidia movement through xylary tissue. Both gums and bubble-like structures occurred in amounts relative to the amount of fungus in all hosts. The bubble-like structures have not been previously reported for oak, but Ouellette (10) observed similar structures in trees with Dutch elm disease. Gagnon (5) speculated that they were some type of polyphenol formed as a result of a pH change from the action of *Ceratocystis ulmi* on the host parenchyma cell. This appears to be a reasonable theory since the bubble-like structures are apparently insoluble in xylary fluid and are found in or near vessels containing the fungus. They may also represent air trapped near areas of colonization.

Tylosis was most intense in white oak. Parenchyma cells of this species appeared to be more readily incited to tylose formation than either red or chestnut oak. This difference in tylose formation may relate to the natural ability of the white oak group to produce more tyloses and further may be an important mechanism of restricting colonization (11).

The striking lack of tyloses in most vessels containing hyphae occurred in all species at all sample times. Therefore, blockage by tyloses in colonized vessels may not be as common as previously thought. This lack of tyloses has been previously noted in diseased elms (7,8). The difference in tylose production suggests that the fungus by its direct or indirect influence may injure or cause the death of those parenchyma cells in close contact with it so that they are incapable of producing tyloses (8). This may be a highly localized phenomenon that has little overall effect on colonization.

In conclusion, it is still difficult to comprehend how colonization is so successful and rapid in red oaks, considering the limited numbers of hyphae and spores observed prior to symptom expression. The answer would seem to lie with the production of small conidia that can be transported rapidly throughout the functional xylem. The greater amount of natural vessel anastomosing (12) and the lack of restriction to lateral growth of *C. fagacearum* must allow for rapid distribution. The ability of the pathogen to rapidly colonize is restricted in white oaks apparently by a more intense and different host response as well as anatomical variability (12). The dark staining reactive zone of parenchyma cells seems to be of major importance to the restriction of the fungus in white oak and would appear to be a prime area for additional study.

LITERATURE CITED

1. BARNETT, H. L. 1953. Isolation and identification of the oak wilt fungus. W. Va. Agric. Exp. Stn. Bull. 359T. 15 pp.
2. BECKMAN, C. H. 1966. Cell irritability and localization of vascular infections in plants. Phytopathology 56:821-824.
3. FEDER, N., and T. P. O'BRIEN. 1968. Plant microtechnique: Some principles and new methods. Am. J. Bot. 55:123-142.
4. FERGUS, C. L., and D. C. WHARTON. 1957. Oak wilt: Histological studies of host reactions and pathogen. Pa. Agric. Exp. Stn. Prog. Rep. 168. 6 pp.
5. GAGNON, C. 1967. Polyphenols and discoloration in the elm disease investigated by histochemical techniques. Can. J. Bot. 45:2119-2124.
6. GREGORY, G. F. 1971. Correlation of isolability of the oak wilt pathogen with leaf wilt and vascular water flow resistance. Phytopathology 61:1003-1005.
7. KRAUSE, C. R., and C. L. WILSON. 1972. Fine structure of *Ceratocystis ulmi* in elm wood. Phytopathology 42:13.
8. MacDONALD, W. L. 1970. Electron microscopy of the elm infected with *Ceratocystis ulmi* (Buism.) C. Moreau. Ph.D. Thesis. Iowa State University, Ames. 161 pp.
9. NAIR, V. M. G. 1964. Pathogenesis of oak wilt in bur oaks. Ph.D. Thesis. University of Wisconsin, Madison. 142 pp. (Univ. Microfilms, Ann Arbor, Mich).
10. OUELLETTE, G. B. 1962. Morphological characteristics of *Ceratocystis ulmi* (Buism.) C. Moreau in American elm trees. Can. J. Bot. 40:1463-1465.
11. PANSHIN, A. J., and C. DeZEEUW. 1970. Textbook of Wood Technology. McGraw-Hill, New York. 705 pp.
12. PARMETER, J. R., J. E. KUNTZ, and A. J. RIKER. 1956. Oak wilt development in bur oaks. Phytopathology 46:423-426.
13. SACHS, I. B., V. M. G. NAIR, and J. E. KUNTZ. 1970. Penetration and degradation of cell walls in oaks infected with *Ceratocystis fagacearum*. Phytopathology 60:1399-1404.
14. STRUCKMEYER, B. E., C. H. BECKMAN, J. E. KUNTZ, and A. J. RIKER. 1954. Plugging of vessels by tyloses and gums in wilting oaks. Phytopathology 44:148-153.
15. STRUCKMEYER, B. E., J. E. KUNTZ, and A. J. RIKER. 1958. Histology of certain oaks infected with the oak wilt fungus. Phytopathology 48:556-561.
16. WILSON, C. L. 1961. Study of the growth of *Ceratocystis fagacearum* in oak wood with the use of autoradiograms. Phytopathology 51:210-215.
17. YOUNG, R. A. 1949. Studies on oak wilt, caused by *Chalara quercina*. Phytopathology 39:425-441.