Isolation of *Ceratocystis fagacearum* from Diseased Live Oak in Texas

by

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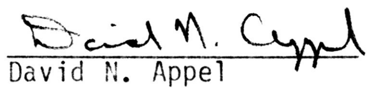
David N. Appel

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ABSTRACT

Five media were compared for their efficiency in isolation of *Ceratocystis fagacearum*, the oak wilt fungus. The efficiency of bole and branch samples was also compared. Potato dextrose agar amended with streptomycin was found to be both efficient and relatively inexpensive when compared to Barnett's medium, which is used most often in isolation of this pathogen. Bole samples were found to be more efficient than branch samples. It is recommended that streptomycin PDA and bole samples be used in the isolation of *C. fagacearum* to improve cost and time efficiency.

C. fagacearum colonized the bole, branches, and roots of inoculated live oak seedlings in just 4 weeks. Attempts were made to quantify the fungus in those seedlings and in naturally colonized trees with suspensions of ground wood tissue. A spermatization technique was also tested to detect the fungus in the wood. Neither attempt was successful. The grinding process, incorporating a Wiley mill, was difficult to keep sterile, resulting in competition by other organisms. It is also possible the fungal conidia were destroyed during the grinding process.

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Isolation of *Ceratocystis fagacearum* from Diseased Live Oak in Texas[a]

Janet E. Steele

INTRODUCTION

Oak wilt, incited by the fungus *Ceratocystis fagacearum* (Bretz) Hunt, causes widespread destruction of live oak (*Quercus virginiana L.*) in central Texas (12). Oak wilt is a common disease of oaks from Minnesota to Pennsylvania and North Carolina to Arkansas (14). Although it has been intensively studied in these areas for 40 years, there are many aspects of the disease that are poorly understood.

Widespread oak mortality of unknown etiology was first reported in Texas in the 1930's, and by the mid-1960's it had been observed throughout most of the natural live oak habitat of Texas (7,10,12). However, *C. fagacearum*, the oak wilt pathogen, was not consistently isolated from diseased trees in Texas until the late 1970's (12). Isolation of the pathogen is difficult because a diseased tree may be invaded by numerous organisms that compete with *C. fagacearum*, thus inhibiting its growth in the laboratory. In addition, a tree must be in early stages of wilt and active colonization for dependable isolation of *C. fagacearum* (1). Without reliable methods for pathogen isolation, a proper diagnosis and recommendations for control cannot be made. For these reasons, more efficient techniques for isolating *C. fagacearum* are needed.

[a] This manuscript was prepared in a manner suitable for publication in Plant Disease.

Oak wilt symptoms on live oak leaves include marginal and veinal chlorosis and necrosis, tip die-back, and defoliation. Trees with advanced disease symptoms exhibit twig and branch dieback, adventitious sprouting on trunks and limbs, reduced leaf growth, and thin crowns. Death of live oaks and Spanish oaks (_Q. texana_ Buckl.) may be rapid, within a matter of weeks once symptoms appear. A small percentage of live oaks may initially survive the disease but eventually decline and die (12).

The means of fungal dissemination and infection courts for _C. fagacearum_ on oaks in Texas are poorly understood. Research in other parts of the country shows that long distance dissemination of the pathogen occurs through animal and insect vectors. The acquisition of inoculum by insects depends upon the formation of sweet-smelling fungal mats underneath the bark of diseased red oaks. The fungal mats are covered with conidia (asexual spores) and ascospores (sexual spores) that are carried by insects to wounds on healthy trees. Ascospore production depends on insects transmitting conidia of one of two sexual compatibility strains (e.g. type A) to a fungal mat of the opposite strain (e.g. type B) (see Figure 1). These mechanisms may also operate in Texas. Live oaks, however, are known to regenerate and grow with common root systems. Therefore, the pathogen could easily be spread into the vascular systems of adjacent and connected trees. This possible means of dissemination needs further investigation.

Control of oak wilt has met with little success. Systemic fungicides are expensive and impractical for large scale use. Removal of diseased trees and sanitary disposal methods are vital in preventing its spread. Amos and True (1) report that the fungus does not survive

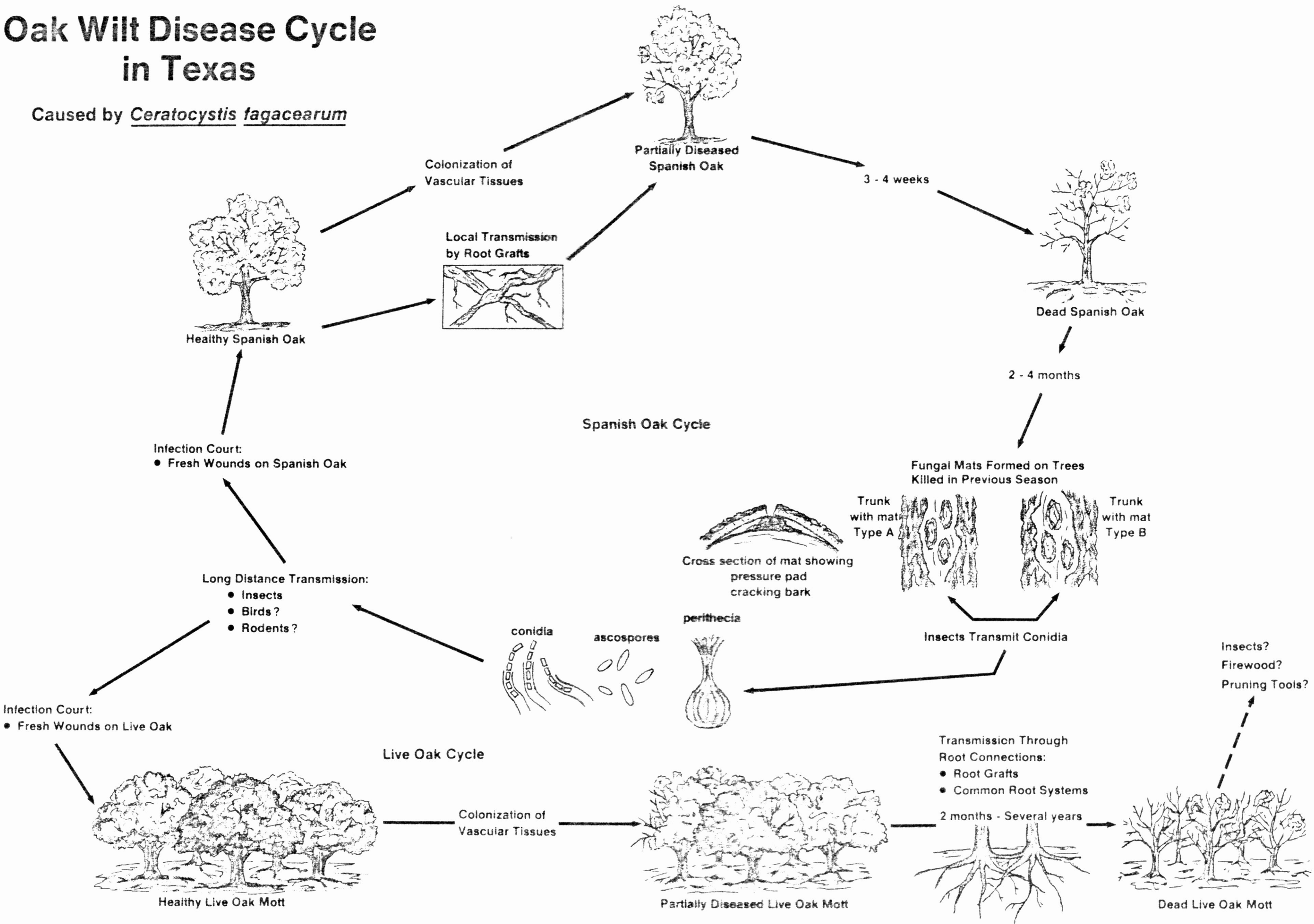

Figure 1. Oak wilt disease cycle in Texas.

longer than 2 years in girdled trees. Trenching has also been used to prevent spread through the common root systems of live oaks.

The objectives of this study were to investigate various isolation techniques for *C. fagacearum*, to identify the location of the pathogen in infected trees, and to follow its spread in inoculated live oak seedlings.

LITERATURE REVIEW

Tsao (20) defines a selective medium as one which can isolate a fungus from its complex natural environment while inhibiting the growth of contaminants and enhancing growth of the desired fungus. Antibiotics are often added to the medium to achieve this selectivity. Schneider (18) compiled a list of 18 antibiotics which inhibit the growth of *C. fagacearum* in an attempt to control oak wilt. Barnett (4) describes a selective medium for the oak wilt fungus which is low in glucose and uses phenylalanine as the sole nitrogen source. Barnett's medium limits the growth of other microorganisms which may be present and supplies biotin which is a necessary supplement for *C. fagacearum*. The pathogen has been isolated on potato dextrose agar (9), various malt agars (19,6), and agars utilizing wood shavings, casamino acids, and cellulose (16). However, Stambaugh (19) reported the Barnett's medium to be superior to these.

Work has also been done to determine the most efficient sampling methods and the effects of temperature on isolating the pathogen. Bretz and Morrison (5) reported difficulty isolating the pathogen after an unusually warm and dry summer. Barnett (2) speculated that prolonged periods of temperatures above 35 C would kill the fungus in the smaller

branches of trees. He also reported that laboratory storage temperatures of 10 C and below were far superior to those above 20 C for survival of the fungus (2). Englerth et al. (8) failed to isolate the fungus from wood with a moisture content below 20%. Clearly, samples must remain cold and moist for efficient isolation. Both branch samples and samples removed from the bole of trees have been used to isolate the fungus, but it is unclear whether one sample type is better than the other. Knighten and Maxwell (11) reported no significant differences in the isolation efficiency of the two, but Merek and Fergus (15) found the survival time of the fungus in bole samples was nearly four times that of branch samples.

Barnett (4) described a method for "rapid" determination of oak wilt. This involved submerging wood chips in a liquid medium, with conidiophores and conidia forming in approximately 5 days. However, isolation of _C. fagacearum_ is often a slow process, generally requiring 2-3 weeks (3). It can also be a frustrating and time-consuming process, as trees with symptoms characteristic of oak wilt may have to be sampled numerous times before positive identification of the fungus can be made (4).

MATERIALS AND METHODS

Isolation Techniques. Trees from Burnet, Texas, exhibiting symptoms characteristic of oak wilt were sampled. Bole samples taken at various heights from 3 trees (A,B, and C) were run on Barnett's medium (Figure 2) and 3 variations of potato dextrose agar (PDA) (Figure 3). These bole samples were first stripped clean of all outer bark and cambium using a sterile chisel and then cut into square pieces approximately 1/2

BARNETT'S MEDIUM

D- glucose	3.0 g
DL-phenylalanine	0.5 g
KH_2PO_4	1.0 g
$MgSO_4 \cdot 7H_2O$	0.5 g
Agar	20.0 g
Microelement solution[a]	1.0 ml
Vitamin solution[b]	1.0 ml
Biotin solution[c]	1.0 ml
H_2O	1.0 l

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[a]Microelement solution yields (per ml):

$ZnSO_4 \cdot 7H_2O$	0.2 mg
$FeSO_4 \cdot 7H_2O$	0.2 mg
$MnSO_4 \cdot 7H_2O$	0.2 mg

[b]Vitamin solution yields (per ml):

Thiamin HCl	1.0 μg

[c]Biotin solution yields (per ml):

Biotin	1.0 μg

MODIFIED BARNETT'S MEDIUM

Increase D-glucose to 5.0 g

Add 20 drops of 50% Lactic Acid solution

Figure 2. Composition of Barnett's and modified Barnett's media.

POTATO DEXTROSE AGAR

Potatoes	200 g
Dextrose	5 g
Agar	17 g
H_2O	1 l

LACTIC ACID PDA

Add 20 drops of 50% Lactic Acid

STREPTOMYCIN PDA

Add 150 mg Streptomycin Sulfate

CYCLOHEXIMIDE PDA

Add 200 mg Cycloheximide

Figure 3. Composition of potato dextrose agar (PDA) and amended PDA.

inch on a side. After surface sterilization in 20% sodium hypochlorite for 60-120 seconds, 4 chips were placed on a petri plate with 5 repititions per medium. The plates were incubated at room temperature up to 4 weeks, and the number of wood chips with *C. fagacearum* growth recorded. An additional tree (D) was sampled using lactic acid-modified Barnett's medium (Figure 2) and streptomycin PDA.

Quantification of C. fagacearum. Wood chips from the bole samples of the trees used in the previous study, as well as wood chips from seedlings inoculated with a "B" strain of the fungus, were ground using a Wiley mill. One gram of this ground tissue was placed in 9 ml of sterile, distilled water. Serial dilutions were made to obtain $1:10^2$, $1:10^3$, $1:10^4$, and $1:10^5$ suspensions of the ground tissue. One ml of these suspensions was pipetted on streptomycin PDA, with 3 repetitions per dilution. The plates were incubated at room temperature and the number of resulting fungal colonies were counted.

In addition, a spermatization technique was used to detect *C. fagacerum* in ground wood tissue removed from inoculated seedlings. One gram of tissue was placed in 9 ml sterile water, and 1 ml of the suspension was pipetted over an "A" strain of the fungus. Eight suspensions were prepared from 2 seedlings, and 3 repititions per suspension were made. The plates were incubated at room temperature and observed for perithecia and ascospore production.

Location of C. fagacearum. A diseased live oak (E) from Burnet was sampled by removing bole samples and primary, secondary, tertiary, and quaternary branch samples (Figure 4). The samples were prepared as described before and placed on modified Barnett's medium and streptomycin PDA. Samples were incubated at room temperature up to 4

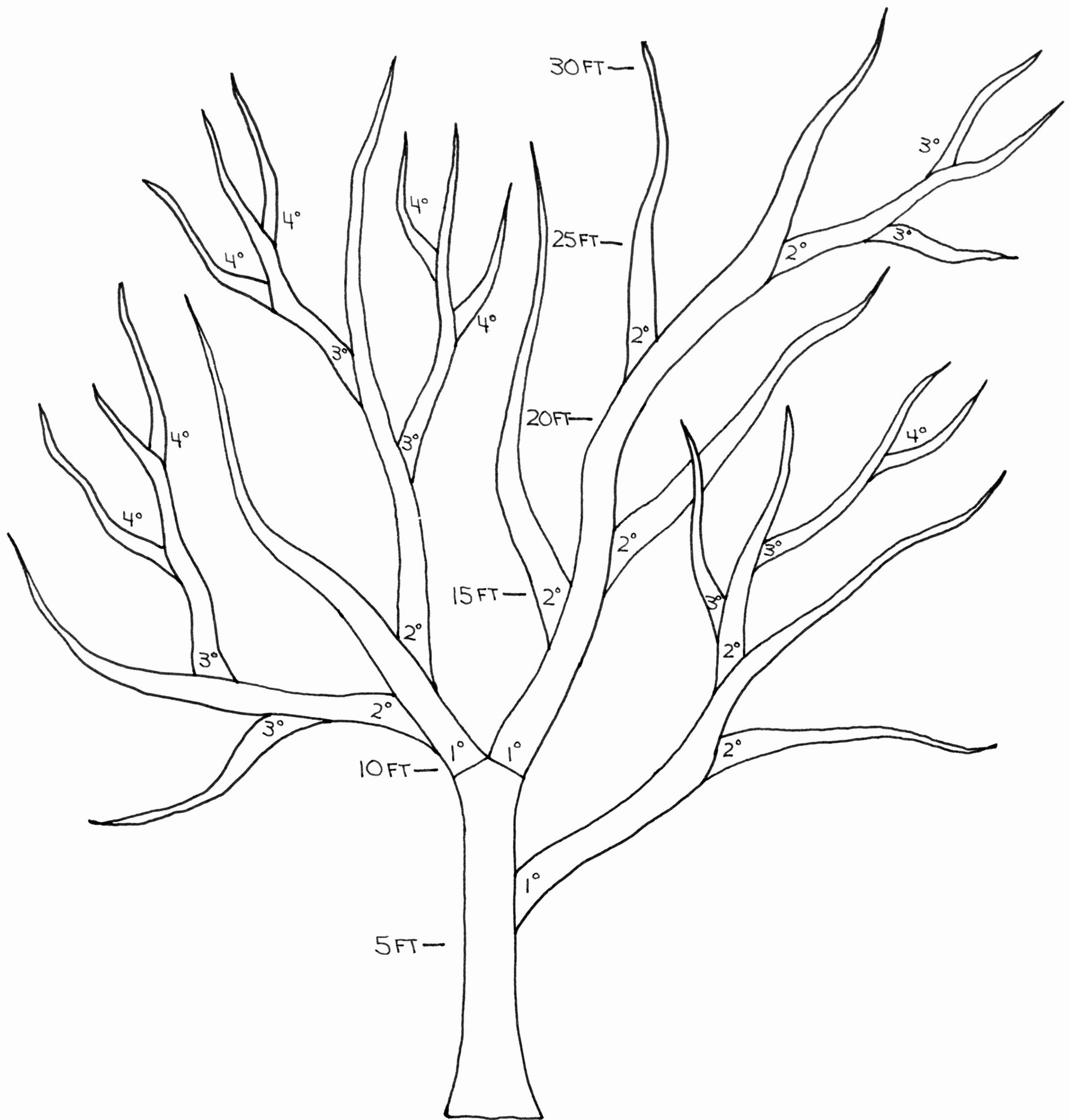

Figure 4. Line drawing of diseased live oak E illustrating sampling positions.

weeks and the number of chips with *C. fagacearum* recorded.

Movement of C. fagacearum. Twelve live oak seedlings approximately 4 feet tall were inoculated with a suspension of *C. fagacearum* obtained by placing plugs of a "B" strain of the fungus into sterile water. The concentration of the inoculum was 600,000 conidia per ml. The main stems of seedlings were wounded using a scalpel, and a few drops of the suspension were placed on the wound. Two trees were inoculated with sterile water as controls. Seedlings inoculated in this manner were also used in other studies (See Materials and Methods, Quantification of *C. fagacearum*).

Inoculated trees were sampled every 4 weeks while the controls were sampled every 8 weeks. A 1-inch section of the trunk surrounding the inoculation site was sampled. Also, sections were removed every third inch above and below the inoculation site. Three roots and at least 4 branches were sampled from each tree. All samples were stripped of bark and cambium, split into 2-4 pieces, surface sterilized in 20% sodium hypochlorite, and plated on streptomycin PDA. The samples were incubated at room temperature and those with *C. fagacearum* growth were recorded. Symptoms on branches were recorded and compared with the isolation results.

RESULTS

Isolation Techniques. During sampling of tree A, *C. fagacearum* was isolated from a total of 23/120 wood chips on the lactic acid PDA, compared to 18/120 for streptomycin PDA and 11/120 for the Barnett's medium (Table 1). No isolates were obtained from the cycloheximide PDA. The frequencies of *C. fagacearum* isolation were much lower for trees B

and C (Tables 2 and 3). Only 6 isolations were obtained from tree B out of 240 total wood chips. Again, there were no isolations on the cycloheximide PDA. No isolations were made from tree C on any of the media used. After evaluating the results from these 3 trees, samples from tree D were run on just two media, modified Barnett's medium and streptomycin PDA (Table 4). On the streptomycin PDA, *C. fagacearum* was isolated from 30/120 wood chips compared to 23/120 on the modified Barnett's medium.

Quantification of C. fagacearum. No isolations of *C. fagacearum* were obtained using the dilution method. Most colonies were *Aspergillus* and *Penicillium* spp. The total number of fungal colonies did decrease as the dilution factor increased.

The spermatization technique also failed to detect the fungus in inoculated seedlings. No fertile perithecia were found on the spermatized plates.

Location of C. fagacearum. Tree E was found to be heavily colonized by *C. fagacearum* (Figure 5). The pathogen was isolated from all bole samples (Table 5) and from primary, secondary, tertiary and quaternary branches on some of the major limb systems (Table 6). On the bole samples, the streptomycin PDA was slightly more efficient than the modified Barnett's medium, with 21/200 isolations on the former compared to 11/200 isolations on the latter. However, the modified Barnett's medium was more efficient than the streptomycin PDA on the branch samples (Table 6).

Movement of C. fagacearum. The pathogen was isolated 27 inches above the inoculation point 4 weeks after inoculation. This rate of colonization corresponds to nearly 1 inch every 24 hours. The pathogen

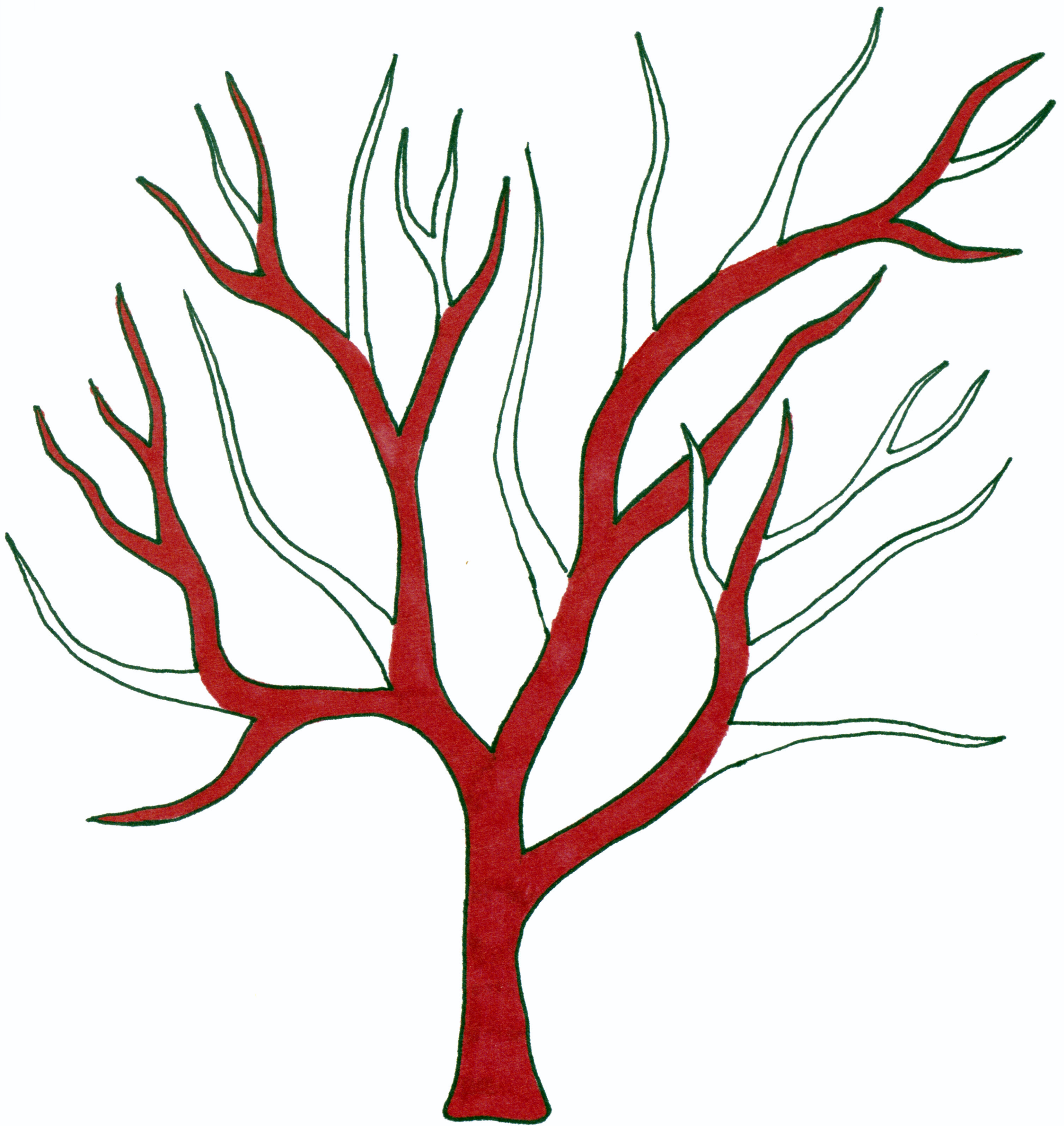

Figure 5. Line drawing of diseased live oak E. Shaded areas indicate isolation of *C. fagacearum*.

was also isolated after 4 weeks from 2 of 7 roots sampled and 1 of 10 branches sampled. At the 12 and 16-week samplings, the pathogen was isolated from 50% of the branches sampled. No isolations from branches were made at the 8-week sampling. Due to competition by other organisms present, including _Trichoderma_ spp., isolation of _C. fagacearum_ from root samples was inhibited at the 8, 12, and 16-week sampling periods. No isolations of _C. fagacearum_ were made from either of the control trees.

Disease symptoms were evident at the 4-week sampling with approximately 25% of the crowns affected. At the 8 and 12-week sampling dates anywhere from 25 to 75% of the crowns were showing symptoms. By the 16-week sampling the old growth on 1 of the 2 remaining trees was nearly gone. But, this tree was producing new, healthy shoots. Over 75% of the crown on the second tree displayed symptoms. The symptoms observed included leaf curling, general discoloration, and defoliation.

DISCUSSION AND CONCLUSIONS

Isolation of _C. fagacearum_ from suspect trees is most efficient when bole samples are utilized. As Figure 5 illustrates, the pathogen was isolated from all bole samples but only 13 of 28 branches. All branches showed typical symptoms of colonization. _Ceratocystis fagacearum_ is heat sensitive (5,15) and can be killed in smaller-diameter branches during hot weather. In general, branch samples showed abundant growth of other organisms, which could inhibit growth and identification of _C. fagacearum_. Also, the pathogen is not necessarily in every branch of an infected tree. For these reasons it is recommended that bole samples be used exclusively for idenfication of _C._

fagacearum. This is especially important for the homeowner who must send samples to professionals for identification. Random branch sampling could result in misdiagnosis or delays. For accurate and efficient diagnosis, bole samples should be utilized.

The goal of the media study was to identify a medium which would be as efficient for isolation as Barnett's medium but less expensive and less tedious to prepare. In the previous study, a PDA medium amended with lactic acid or streptomycin was as equally useful in isolation of *C. fagacearum* as Barnett's medium. The PDA medium is also relatively inexpensive and simple to prepare.

C. fagacearum moved much more quickly in the inoculated seedlings than expected. The study was set up to last 16 weeks, and after 4 weeks the pathogen was isolated from the top bole samples of some seedlings as well as branch and root samples. This emphasizes the need for rapid identification of the pathogen during initial stages of colonization if attempts are to be made to save the tree. Delays in identification will simply give the pathogen time to colonize more of the tree.

The quantification and perithecia studies both involved use of ground tissue using the Wiley mill and dilutions of this tissue. Neither study was successful. Competition by other organisms was a problem as sterile conditions using the Wiley mill were impossible to maintain. The viability of fungal conidia may have been diminished in the grinding process. More work is necessary in this area to determine if the dilution technique could be useful in further studies involving *C. fagacearum*.

TABLE 1. Success in isolation of oak wilt fungus (No. *Ceratocystis fagacearum* isolations/no. wood chips) from diseased live oak A[a].

Bole sample height (ft)	Isolation Frequency (No. *C. fagacearum* isolations/no. wood chips)			
	Barnett's Medium	Lactic Acid PDA	Streptomycin PDA	Cycloheximide PDA
1	2/20	8/20	6/20	0/20
1.5	1/20	2/20	2/20	0/20
3	4/20	0/20	2/20	0/20
3.5	1/20	7/20	3/20	0/20
4.5	3/40	6/40	5/40	0/40

[a]Tree A was located in Burnet, Texas, and was sampled on September 1, 1983.

TABLE 2. Success in isolation of oak wilt fungus (No. *Ceratocystis fagacearum* isolations/no. wood chips) from diseased live oak B[a].

Bole sample height (ft)	Isolation Frequency (No. *C. fagacearum* isolations/no. wood chips)			
	Barnett's Medium	Lactic Acid PDA	Streptomycin PDA	Cycloheximide PDA
1	0/20	0/20	2/20	0/20
2	2/20	2/20	0/20	0/20
4	0/20	0/20	0/20	0/20

[a]Tree B was located in Burnet, Texas, and was sampled on October 13, 1983.

TABLE 3. Success in isolation of oak wilt fungus (No. *Ceratocystis fagacearum* isolations/no. wood chips) from diseased live oak C[a].

Bole sample height (ft)	Isolation Frequency (No. *C. fagacearum* isolations/no. wood chips)			
	Barnett's Medium	Lactic Acid PDA	Streptomycin PDA	Cycloheximide PDA
1	0/20	0/20	0/20	0/20
2	0/20	0/20	0/20	0/20
4	0/20	0/20	0/20	0/20

[a]Tree C was located in Burnet, Texas, and was sampled on October 13, 1983.

TABLE 4. Success in isolation of oak wilt fungus (No. *Ceratocystis fagacearum* isolations/no. wood chips) from diseased live oak D[a].

Bole sample height (ft)	Isolation Frequency (No. *C. fagacearum* isolations/no. wood chips)	
	Modified Barnett's Medium	Streptomycin PDA
1	11/40	7/40
3	10/40	17/40
4	2/40	6/40

[a]Tree D was located in Burnet, Texas, and was sampled on November 10, 1983.

TABLE 5. Success in isolation of oak wilt fungus (No. *Ceratocystis fagacearum* isolations/no. wood chips) from the bole of diseased live oak E[a].

Bole sample height (ft)	Isolation Frequency (No. *C. fagacearum* isolations/no. wood chips)	
	Modified Barnett's Medium	Streptomycin PDA
1	0/40	4/40
3	4/40	12/40
5	2/40	0/40
7	3/40	3/40
9	2/40	2/40

[a]Tree E was located in Burnet, Texas, and was sampled on November 18, 1983.

TABLE 6. Success in isolation of oak wilt fungus (No. *Ceratocystis fagacearum* isolations/no. wood chips) from the branches of diseased live oak E[a].

Branch sample type	Isolation Frequency (No. *C. fagacearum* isolations/no. wood chips): Modified Barnett's Medium	Streptomycin PDA
Primary	3/100	10/100
Secondary	21/340	10/340
Tertiary	7/360	3/360
Quaternary	17/300	5/300

[a]Tree E was located in Burnet, Texas, and was sampled November 18, 1983.

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