# Isolation of *Ceratocystis ulmi* from Deep Annual Rings of Elms in California

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**ABSTRACT**

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The Dutch elm disease fungus, *Ceratocystis ulmi*, has been isolated from very old annual rings carefully dissected from numerous trees of various elm species in California. These included a 27-yr-old (1953) ring of an American elm (*Ulmus americana*). The fungus was isolated from rings at least 7 yr old in six different counties in the area around San Francisco Bay. However, the ability of the fungus to move laterally in a tree makes it difficult to determine how long such a tree has actually been infected.

In the laboratory of the California Dutch elm disease (DED) project, samples from removed trees that are confirmed positive for DED are frequently examined for discoloration present in annual rings older than that of the current year. In 1978, this procedure was done infrequently; the results were so intriguing, however, that by the 1980 season it was being done regularly with all samples of elm diagnosed positive for DED.

The objective of this annual ring study was to determine the age of infection of individual trees as well as to get an indication of the length of time DED has been present in particular geographic areas. The correlation of depth of DED discoloration with age of infection has been studied by Campana et al (3) and by Banfield (1,2).

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## MATERIALS AND METHODS

**Host species.** Samples of 160 elms diagnosed positive for DED, submitted over the 3-yr period 1978 through 1980 and examined for deep DED discoloration, included those from *Ulmus americana* L. (American elm) (8.8% of total), *U. pumila* L. (Siberian elm) (15.6%), *U. parvifolia* Jacq. (Chinese elm) (5.6%), *Zelkova serrata* (Thunb.) Mak. (Japanese zelkova) (1.3%), and several species of "European elms" (68.7%). Elms that fell into the European elm category included such species as *U. glabra* Huds., *U. carpinifolia* Ruppius ex Suckow, *U. procera* Salisb., and many others, including numerous hybrids. Data on the various elm species that comprise the European elm population in California were not available.

**Elm extract agar.** Elm extract agar was prepared from healthy American elm wood collected outside the DED quarantine area. Branches 2–3 cm in diameter were chipped, and 400 g of the chips were boiled in 3 L of distilled water for 30 min. The "elm broth" that resulted was maintained at 80 C for an additional 2 hr, after which additional distilled water was added to bring the volume to 3.2 L. The elm broth was strained through cheesecloth, Bactoagar was added to a 2% level (w/v), and the mixture was autoclaved 20 min at 121 C.

**Septic cultures.** Septic cultures were prepared by placing sterilized seed blotter squares (steel-blue Anchor seed germination blotter 7530-999-9999-0, Anchor Paper Co., St. Paul, MN) measuring 5 × 5 cm in sterile plastic petri plates. The seed blotters were saturated with sterile distilled water. Wood samples with only freshly exposed surfaces (but not surface sterilized) were placed in the petri plates, which were double-sealed with paraffin tape to maintain humidity and to prevent contamination via mites. Control cultures were prepared in the same manner, but sterilized American elm chips were substituted for the wood samples.

**Sample processing.** Samples generally consisted of cross sections 5–10 cm thick and from 20 to 80 cm in diameter taken from various parts of the removed trees. Such samples nearly always included a trunk cross section.

A sharp knife or chisel was used to remove a portion of the surface of a wood cross section. The freshly exposed surface was then examined with a hand lens or dissection microscope so that the exact age of the annual rings could be determined.

Portions of individual annual rings showing discoloration were removed with a chisel that was sterilized between each cut by being dipped in ethanol and flamed. The cross section was split to expose a longitudinal surface from which a portion of the discolored ring was removed. The cross section surfaces of the pieces were also removed with the sterilized chisel so that only fresh wood surfaces were exposed. Where the DED discoloration was clear of other discoloration, such as that caused by wetwood or by other organisms, the discolored portions were plated on elm extract agar (EEA). At least one septic culture (5) was made for every sample, whether discoloration was separate from or obscured by discoloration from other organisms.

Plates were labelled with the year of the annual ring from which the discolored wood was taken. Cultures were incubated at room temperature and exposed to both room and natural light. Cultures were examined for the presence of *Ceratocystis ulmi* (Buism.) C. Moreau at 4- to 5-day intervals for the first 2 wk and every 2 wk thereafter for a period of up to 2 mo or until the pathogen appeared in the culture.

**Mating type and aggression tests.** Single-spored cultures of *C. ulmi* of known mating types were grown on sterilized American elm wood (same as that used for EEA), then transferred to EEA. A mass transfer of an A mating type (ME 37, from Maine) or a B mating type (Mass 14, from Massachusetts) was placed on a 0.5-mm-thick, 4- to 6-cm-diameter, sterilized American elm disk along with an isolate to be tested in a procedure similar to that of Shafer and Liming (8). The control cultures were supplied by Dr. Richard Campana of Orono, ME. The mating test cultures were incubated at 17 C and received 12 hr of fluorescent light per day. After 4 wk, the cultures were examined for the presence of *C. ulmi* perithecia and ascospores. Controls consisted of known A mated with known B, of known A grown alone, of known B grown alone, and of the test isolates grown alone.

Aggression tests were conducted according to the procedure of Gibbs and Brasier (6), using 2% Oxoid malt agar. Test cultures were examined after 7 days and compared with controls. Control cultures were the same as those used for mating type testing. ME 37 was an aggressive isolate and Mass 14 was a nonaggressive isolate.

## RESULTS

Viable *C. ulmi* was frequently isolated from "old" annual rings, ie, 5 or more years old. *C. ulmi* was more frequently isolated from old annual rings of Siberian elms over the 3 yr of the study than from those of other elm species, and the mean age of the oldest positive rings of Siberian elms was 5 yr or more throughout the study (Table 1). The oldest *C. ulmi* positive annual ring (27 yr) was in an American elm in San Mateo County, which also had a positive 1980 annual ring. Both the 1953 and 1980 isolates were tested for aggressiveness and for mating type. Both isolates were typical aggressive isolates of the B mating type. All other California isolates of *C. ulmi* tested (more than 200 from various host species, including zelkova) have also been typical aggressive isolates of the B mating type.

San Mateo County was the site of the oldest *C. ulmi* positive annual ring of any species found throughout the study, although in Sonoma County a 14-yr-old *C. ulmi* positive ring was found in 1979 (Table 2). With the exception of Solano and Alameda counties, in each of which only one positive tree was sampled for examination in 1980, every county in the

**Table 1.** Age of annual rings discolored by Dutch elm disease from different elm species in California

| Host species | No. of trees with discoloration[a] | | | Age of oldest infected ring[b] | | | Mean age of oldest infected rings[c] | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1978 | 1979 | 1980 | 1978 | 1979 | 1980 | 1978 | 1979 | 1980 |
| Siberian | 3 | 11 | 11 | 11[d] | 14 | 12 | 5[e] | 5 | 6 |
| European | 19 | 39 | 52 | 6 | 14 | 15 | 3 | 2 | 3 |
| American | 5 | 4 | 5 | 4 | 4 | 27 | 3 | 2 | 8 |
| Chinese | 3 | 2 | 4 | 2 | 5 | 3 | 2 | 4 | 1 |
| Zelkova[f] | 2 | 0 | 0 | 1 | ...[g] | ... | 1 | ... | ... |

[a] In annual rings older than that of the current year.
[b] Including all trees from which *Ceratocystis ulmi* was isolated.
[c] Including all samples of each listed species from which *C. ulmi* was isolated in 1978, 1979, or 1980.
[d] Age of oldest infected ring at the time of examination.
[e] Mean age of the "oldest" infected annual rings from all trees examined in this group.
[f] *Zelkova serrata*, although not a species of *Ulmus*, is also a naturally infected host of Dutch elm disease in California.
[g] No samples submitted.

**Table 2.** Age of annual rings discolored by Dutch elm disease from different California counties

| County | No. of trees with discoloration[a] | | | Age of oldest infected ring[b] | | | Mean age of oldest infected rings[c] | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1978 | 1979 | 1980 | 1978 | 1979 | 1980 | 1978 | 1979 | 1980 |
| San Mateo | 12 | 27 | 36 | 11[d] | 14 | 27 | 4[e] | 3 | 5 |
| Santa Clara | 4 | 6 | 9 | 3 | 8 | 13 | 2 | 4 | 4 |
| Napa | 4 | 5 | 4 | 3 | 4 | 7 | 2 | 3 | 6 |
| Sonoma | 12 | 8 | 6 | 6 | 14 | 11 | 3 | 4 | 4 |
| Contra Costa | 0 | 4 | 5 | ...[f] | 7 | 12 | ... | 6 | 6 |
| Marin | 0 | 4 | 11 | ... | 4 | 11 | ... | 1 | 4 |
| Solano | 0 | 0 | 1 | ... | ... | 2 | ... | ... | 2 |
| Alameda | 0 | 1 | 0 | ... | 1 | ... | ... | 1 | ... |

[a] In annual rings older than that of the current year.
[b] Including all trees from which *Ceratocystis ulmi* was isolated.
[c] Including all samples of each listed species from which *C. ulmi* was isolated in 1978, 1979, or 1980.
[d] Age of oldest infected ring at time of examination.
[e] Mean age of the "oldest" infected annual rings from all trees examined in this group.
[f] No samples submitted.

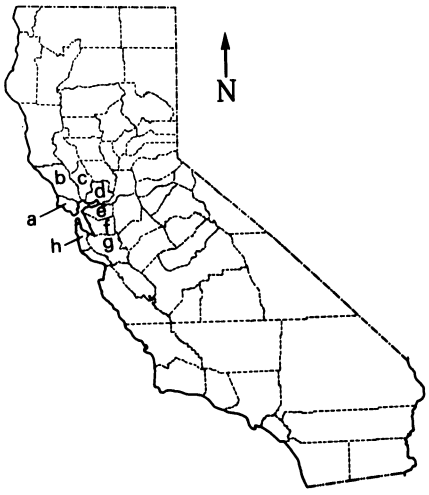


**Fig. 1.** California counties from which infected elms were sampled for examination of deeply buried infections. a = Marin, b = Sonoma, c = Napa, d = Solano, e = Contra Costa, f = Alameda, g = Santa Clara, h = San Mateo.

California DED project area had elm trees with *C. ulmi*-positive buried annual rings (Fig. 1). The ages of the deepest infected rings in these counties ranged from 7 yr (Napa) to 27 yr (San Mateo).

No control cultures yielded *C. ulmi*.

## DISCUSSION

In 1979, two elm trees yielded positive cultures of the DED fungus from 14-yr-old annual rings. These two trees appear to have been infected for the first time in 1965, whereas a previous report of a positive culture isolated from a 14-yr-old annual ring in 1977 (7) would represent a tree presumably infected for the first time in 1963. The 1980 cultures from a 27-yr-old ring of an American elm from San Mateo County suggest that infection originally occurred in that tree in 1953. Two additional trees, both European elms from San Mateo County, also yielded cultures in 1980 from the 1965 annual ring. Clearly, deep discoloration from apparently old infections is common in California. But how do these deep infections occur, and how do they relate to the actual age of infection?

The 1953 and 1980 ring isolates from the American elm mentioned above were both found to be typical examples of aggressive isolates of the B mating type. Aggressive isolates of *C. ulmi* may have been present in the United States as early as 1937 when Walters (9) published photographs of what appeared to be aggressive isolates grown on malt agar. In the same report, Walters also made mention of a "perithecial isolate" of *C. ulmi* from Norfolk, VA, so it can be assumed that the B mating type had also been in the United States at least 16 yr earlier than 1953. Even though the 1953 and 1980 annual rings were 27 yr apart, one cannot rule out the possibility that both the 1953 and 1980 isolates arose from a common infection, possibly in either year.

The lateral movement of the DED fungus in a host has been studied by Campana and Hyland (4) and in great detail by Banfield (1,2). Studies by Campana and Hyland (4) strongly suggest that lateral movement of DED fungus propagules is likely to occur in the roots of an elm rather than in the trunk or branches. Intervessel distances in elm stems were significantly greater than those in roots. Banfield (2) noted that adjacent annual rings have many vessels and tracheids in contact at the periphery of each annual ring. Furthermore, from his experiments, in which he sectioned roots of inoculated trees at 2.4-cm intervals, he observed that discolored vessels of one annual ring were in direct contact with discolored vessels of preceding annual rings in one to several points. Thus, the root is an ideal site for crossover of the fungus from one annual ring to another.

Banfield also reported the presence of *C. ulmi* in ray parenchyma and documented it with photomicrographs (2). This, too, could be a possible way for the DED fungus to move laterally. Throughout our study, discolored vascular rays were occasionally observed to be associated with wetwood discoloration, although not with DED discoloration.

Throughout this study, apparent lateral movement of wetwood staining was frequently associated with wounds, particularly wounds caused by chemical injection equipment. In addition, in a concurrent study, several American elms inoculated with the DED fungus for pathogenicity experiments exhibited DED discoloration in one to two annual rings older than the current year's growth only 2 mo after inoculation. This, too, may have been the result of the inoculation wound intersecting more than one layer of xylem. Thus, wounding may also play a role in some deeply buried infections.

Viable DED fungus was isolated from annual rings up to 27 yr old. However, considering the possible lateral movement of the fungus, it is difficult to draw conclusions as to how long such trees have actually been infected.

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