Phytophthora cinnamomi as a Cause of Oak Mortality in the State of Colima, Mexico

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ABSTRACT

Tainter, F. H., O'Brien, J. G., Hernández, A., Orozco, F., and Rebolledo, O. 2000. *Phytophthora cinnamomi* as a cause of oak mortality in the state of Colima, Mexico. Plant Dis. 84:394-398.

This research identifies the root pathogen *Phytophthora cinnamomi* as the primary cause of mortality in a 300-ha disease center of mixed oak trees in a native forest in southern Mexico. In increasing order of apparent field resistance to the disease, the major oak species are *Quercus glaucoides*, *Q. peduncularis*, and *Q. salicifolia*. *P. cinnamomi* was isolated from soil in the affected area from symptomatic trees and was successfully used to perform Koch's postulates on these three oak species. Artificial and natural infections produced vertically elongated discolorations in the outer xylem and distinctive phloem canker lesions with a sharp demarcation line between healthy and affected tissues. In *Q. glaucoides* there is little evidence that this oak species is able to resist the girdling effects of the phloem lesions, but in *Q. peduncularis*, and especially in *Q. salicifolia*, increased production of callus tissue around the phloem canker lesions suggests an active resistance mechanism that may allow these infected trees to survive somewhat longer. This particular incident is unlike other recent reports in other parts of the world of oak mortality caused by *P. cinnamomi* because the initial appearance of disease in this area is known (just prior to 1987), and it has subsequently expanded to the present area of 300 ha (in 1999) as a distinctive infection locus with periodically advancing infection fronts. This incident is also another dramatic illustration of the potential environmental damage that can result when *P. cinnamomi* is introduced into a simple forest ecosystem where the major overstory trees are susceptible to infection and are killed.

Additional keywords: introduced pathogen, root disease

A severe outbreak of oak mortality was first observed in 1987 in the ejido of El Arrayanal near the city of Colima, state of Colima, and 560 km west of Mexico City (Fig. 1A and B). An ejido is a kind of land-based cooperative, established under federal guidance, in which the residents collectively own the land and work with pooled resources. In this ejido, subsistence agriculture is the primary activity, with charcoal production used to generate capital.

Because of the increasing size of the affected area, in 1991, representatives of the ejido requested technical assistance from the Secretaría de Medio Ambiente, Recursos Naturales y Pesca (SEMARNAP) staff

located in the city of Colima. In 1992, Mexican authorities requested technical assistance to determine the cause of the mortality. By that time, the mortality had spread southwesterly and affected about 50 ha of oak forest (Figs. 1 and 2A). SEMARNAP authorities were especially concerned that the oak wilt pathogen, *Ceratocystis fagacearum* (Bretz) Hunt, may have spread into Mexico from Texas, where oak wilt had increased in importance since the 1960s. Surveys in the area were made in 1992, 1993, 1994, 1995, and 1997. The mortality continued to spread west and east, and the affected area grew to 150 ha in 1993 and to 180 ha in 1994. By 1995, the mortality had reached the edge of the forest in the west and north of the village of El Arrayanal but continued to spread eastward. The affected area had increased to 200 ha, and a great deal of oak tree mortality had occurred. The mortality abruptly stopped spreading toward the south at the boundary between the oak-dominated forest and a mesic tropical hardwood forest. By 1997, the affected

area had increased to at least 300 ha, and it is presently spreading eastward into unaffected oak forest, progressing both uphill and across streams. The terrain is hilly and very rocky, and the shallow soils are heavy clays.

The three oak species prevalent in the area are *Quercus glaucoides*, *Q. peduncularis*, and *Q. salicifolia*. With the onset of symptoms, *Q. glaucoides* declines rapidly and within several weeks to several months dies, whereas *Q. salicifolia* takes months, or more usually, several years to die after the first appearance of foliar symptoms. The behavior of *Q. peduncularis* is intermediate between the other two oak species. Nance (*Byrsonima crassifolia*), a shrubby understory species, disappeared from the affected area during the early part of the mortality episode.

Symptoms on affected oaks include foliar interveinal chlorosis and necrosis (Fig. 2B), wilt, dieback, and discolorations in the phloem-xylem. Foliar discoloration is transitory, and the leaves are either cast or necrose and remain attached for a time after the tree dies. Despite several attempts, the oak wilt pathogen, *C. fagacearum*, has not been isolated from symptomatic trees.

Residents described their initial observations on the mortality as a moving front that originated near a residence in the village and moved first toward the southwest for several years, then moved west and east for several more years, and then finally moved only toward the east (Fig. 1). In the first two directions, the front stopped when it confronted a change in either soil type or vegetation. Presently, the front is moving toward a vast natural oak forest composed largely of the three oak species known to be susceptible to the mortality. There do not appear to be any obstacles in the path of the expanding epiphytotic to the east.

During the 1995 survey, bleeding cankers were observed on several of the affected oaks. After removal of the outer bark, a distinctive canker-like symptom consisting of dark red phloem delimited by a black zone line was observed. The cankers resembled cankers of ink disease pro-

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Accepted for publication 7 December 1999.

Publication no. D-2000-0131-02S
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duced on North American oaks inoculated with the root pathogen *Phytophthora cinnamomi* Rands (4). In 1997, we attempted to isolate specific primary root pathogens (including *P. cinnamomi*) and determine their possible involvement in the oak mortality.

MATERIALS AND METHODS

Field survey. A total of 19 soil samples of approximately 20 g each were collected from six sample points (Fig. 1B) within the decline area in July 1997, each sample from the vicinity of a tree (15 total) with visible foliar symptoms. The samples were divided in half, with half subsequently analyzed in Mexico and half at Clemson University. The same isolation procedure was followed at both locations. Soil dilu-

tions were based on the procedure of Hornby and Ullstrup (3), except that 0.2% water agar was used in place of 1% sodium carboxymethyl cellulose as the diluent. Plating was on pimarinic-chloramphenicol-hymexazol (PCH) selective medium (10). All plates were read 3 weeks after plating, colonies of Pythiaceae fungi were subcultured, and tentative identifications were made.

Greenhouse inoculation trial. Six 12-month-old seedlings of *Q. glaucoides* grown in 1-gal pots in Fafard no. 3F potting medium were inoculated with a 3-mm agar plug of the Mexican isolate of *P. cinnamomi* (designated Mex. 12) grown for 14 days on acidified potato dextrose agar in a small wound created on a major root by slicing through the bark into the xylem

to create an 8-mm-long flap of bark and outer xylem. Three additional seedlings were similarly wounded but inoculated only with a sterile agar plug. All inoculation sites were wrapped with several layers of Parafilm, and the seedlings were replanted in the pots. Plants were incubated for 18 days, observed for symptoms, and tissue chips from the inoculated areas, and resulting lesions, were plated onto PCH medium.

Field inoculation trials. Twelve apparently healthy trees each of *Q. glaucoides*, *Q. peduncularis*, and *Q. salicifolia* were located in the oldest part of the affected area west of the village of El Arrayanal and inoculated on 14 October 1998. This date was near the end of the wet season, which begins in June and ends in

Fig. 1. (A) Map of Mexico, showing approximate location of oak mortality; (B) Map of the political boundaries of the ejido of El Arrayanal, showing approximate extent of oak mortality (dotted line) and plot locations (open circles); and (C) Map of oak mortality, showing village of El Arrayanal, permanent watercourses (solid lines), seasonal watercourses (dashed lines), and approximate locations of advancing oak mortality (arrows) from 1987 through 1997. The continuous dotted line marks the present boundary of the oak mortality area. The shorter dotted lines with arrows indicate the approximate advancing front of oak mortality during a particular year.

November. The trees were randomly selected. They were scattered, as they were among the few asymptomatic trees remaining in the area. Average diameters (cm) at 1.4 m and total heights (m) for the three oak species inoculated were: *Q. glaucoides*: 25.0/8.9, *Q. peduncularis*: 21.9/8.6, and *Q. salicifolia*: 26.7/12.2, respectively. While there were no significant differences in trunk diameters, *Q. salicifolia* trees were significantly taller than the other two species.

Bark plugs were removed from the trees at a height of 1.4 m with a 1.5-cm-diameter punch. Agar plugs, 1.2 cm diameter, of the Mexican isolate (Mex. 12) of *P. cinnamomi* grown on 14-day-old cultures of acidified potato dextrose agar were then inserted into the wounds, one plug per wound, so that the mycelium was in contact with the cambium. The bark plugs were replaced and covered with a 6-cm square of steril-

ized cotton and a 10-cm square of aluminum foil. The entire inoculated area was then covered with two to three layers of duct tape. A disk of sterile agar in a second wound approximately 15 cm to the right of the first inoculation point served as the control so that the reaction of each tree to wounding alone could be observed.

On 2 to 3 February 1999, 111 days after the trees were inoculated, the inoculated areas were debarked so that the entire resulting phloem lesion was visible. This date was approximately halfway through the dry season, and the trees were dormant, with no foliage. The length and width of phloem lesions were recorded. The phloem was then shaved off to reveal discolored xylem tissue, and the length and width of those discolorations were also recorded.

Wood chips from the phloem canker margin of 21 inoculated trees and from bleeding cankers on five adjacent non-

inoculated trees of *Q. salicifolia* were removed immediately after measurements were taken. The chips were placed directly onto PCH medium and monitored for fungal growth. Chips were not removed from the discolored xylem for culturing.

The opposite face of each tree was inoculated in the same manner as in the October inoculations with the same isolate of *P. cinnamomi*. On 14 to 15 June 1999, 133 days after the trees were inoculated for the second time, resulting cankers were observed and measured as described above, with the addition that chips of discolored xylem tissues were also removed for culturing. All data were analyzed using ANOVA, Tukey's Studentized range test, and Duncan's multiple range test (8).

RESULTS

Most of the soil dilution plates prepared in Mexico yielded cultures of *Trichoderma*

Fig. 2. Views of *Phytophthora cinnamomi*-caused oak mortality in Mexico. (A) Aerial photograph of portion of oak mortality area; (B) foliar discoloration on *Quercus peduncularis*; (C) phloem canker on *Quercus glaucoides*; (D) phloem canker on *Quercus peduncularis*; and (E) phloem canker on *Quercus salicifolia*.

spp., which quickly overgrew the plates, and no isolates of either *Pythium* or *Phytophthora*. Difficulties encountered in maintaining proper temperature control during preparation of the selective medium may have impaired the antibiotic activity, which then favored growth by *Trichoderma*. Of the 39 fungal isolates retrieved from the soil dilutions from Clemson and placed in pure culture, 1 was lost, 10 were determined to be *Pythium*-group 1, 27 were *Pythium*-group 2, and 1 was *P. cinnamomi*, from plot 4. Four additional cultures of what were tentatively identified as *P. cinnamomi* were overgrown by contaminants and lost.

Preliminary root-dip inoculations in liquid cultures of representatives from both *Pythium* groups and the surviving Mexican isolate (Mex. 12) of *P. cinnamomi* on 60-day-old seedlings of nine oak species (*Quercus alba*, *Q. palustris*, *Q. prinus*, *Q. velutina*, *Q. phellos*, *Q. coccinea*, *Q. rubra*, *Q. bicolor*, and *Q. pedunculata*) revealed that only the *P. cinnamomi* isolate produced lesions on the roots of inoculated seedlings, which coincidentally also killed most of the seedlings. Previous to and following inoculation, the seedlings were maintained in a tissue culture room at 23 ± 2°C under ambient lighting (3 ± 1 µmol) with cool-white fluorescent lights and a 12-h photoperiod. Only the Mexican isolate (Mex. 12) of *P. cinnamomi* was used in the greenhouse and field inoculations.

Greenhouse inoculation trial. Inoculation of the six seedlings of *Q. glaucooides* with the Mexican isolate (Mex. 12) of *P. cinnamomi* did not produce visible crown symptoms, but in each case, a necrotic lesion 21 mm in average length developed around the inoculation site on each root. As seen in cross-section, the cambium was not girdled but the necrotic area was in the form of a wedge with the widest portion at the cambium (and occupying approximately one-third of the circumference of the cambium), tapering down to a point at the pith. From 60 to 80% of the chips yielded *P. cinnamomi*, with no positive isolations from the control seedlings.

Field inoculation trials. One tree of each species was lost or vandalized before readings could be obtained. Approximately one-third of the inoculated *Q. peduncularis* trees and all of the *Q. salicifolia* trees inoculated in October exhibited evidence of dark reddish black sap bleeding from around the inoculation sites. Removal of the outer bark revealed a canker lesion in the phloem of every inoculated tree (Table 1). While canker width was not significantly different among the three species, average phloem canker lengths on *Q. glaucooides* (30.1 cm) and *Q. peduncularis* (29.4 cm) were significantly greater than on *Q. salicifolia* (10.6 cm).

The inner bark of *Q. glaucooides* is thick and fibrous, similar to the inner hull tissues of a coconut palm fruit, and the cankered tissue changed to a light brown color upon

exposure to the air. The phloem canker margin was detectable within a few seconds after removal of bark but then became barely visible as a dark but not very distinctive line (Fig. 2C). Cankers on *Q. peduncularis* and *Q. salicifolia* were a bright red to dark red with a distinctive black margin (Fig. 2D and E).

Only 6% of the chips taken from canker margins on *Q. glaucooides* yielded *P. cinnamomi*, whereas 44 and 40% were posi-

tive from *Q. peduncularis* and *Q. salicifolia*, respectively. Of the isolations from the five noninoculated, but naturally infected, *Q. salicifolia* trees with bleeding cankers, 83% of the chips from phloem lesions yielded *P. cinnamomi*. These five *Q. salicifolia* trees had phloem cankers identical in appearance to those on the artificially inoculated trees (Fig. 3A and B).

Removal of the phloem and some of the outer xylem revealed a dark discoloration

Table 1. Canker growth in Mexican oak trees (*Quercus* spp.) resulting from inoculations in October 1998 and February 1999 with a Mexican isolate (Mex. 12) of *Phytophthora cinnamomi*

Oak species ^y	Phloem canker length (cm)		Xylem stain length (cm)	
	October	February	October	February
<i>Q. glaucooides</i>	30.1 aB ^z	14.2 aB	45.5 aA	24.3 bA
<i>Q. peduncularis</i>	29.4 aA	19.7 aA	23.0 bA	15.9 bA
<i>Q. salicifolia</i>	10.6 bA	26.8 aA	15.0 bA	80.4 aB

^y Twelve trees of each species were inoculated; however, one tree of each was lost, so data reflect 11 trees of each species.

^z Means within a column followed by the same lowercase letter are not different at *P* = 0.05. Means within a row followed by the same uppercase letter are not different at *P* = 0.05.

Fig. 3. Natural infection of *Phytophthora cinnamomi* in *Quercus salicifolia*. (A) External view of bleeding cankers, and (B) internal view of phloem cankers on same tree.

that extended well above and below the inoculation point (Table 1). In *Q. glaucooides*, this stain length averaged 45.4 cm, significantly greater than the 23.0 and 15.0 cm for *Q. peduncularis* and *Q. salicifolia*, respectively. The discoloration was 2.6 cm wide in *Q. salicifolia*, significantly less than for the other two species. Whether xylem discoloration represents only a host reaction to phloem colonization, or is also colonized, is not known, because isolations were not made from these tissues.

In the February inoculations, phloem canker lengths were 14.2 cm for *Q. glaucooides*, 19.7 cm for *Q. peduncularis*, and 26.8 cm for *Q. salicifolia*, but there was no statistical difference among the three oak species. Xylem stain lengths were 24.3 cm for *Q. glaucooides*, 15.9 cm for *Q. peduncularis*, and 80.4 cm for *Q. salicifolia* (significantly greater). There were no differences in phloem canker width or in xylem stain width among the three oak species.

Chips removed from phloem canker margins yielded an average of 35% positive isolations and an average of 6% positive isolations from discolored xylem tissues from all three oak species.

DISCUSSION

This research is significant for two reasons. The most important is that the results prove that *P. cinnamomi* is the primary cause of oak mortality in this forest in Mexico. This is also the first report of susceptibility of these three oak species to *P. cinnamomi*. Koch's postulates were performed first on a small scale in the greenhouse and then on a larger scale in the field with nonsymptomatic trees within the affected site. *P. cinnamomi* was recovered not only from inoculated trees, but also from symptomatic naturally infected *Q. salicifolia* trees.

The multitude of secondary organisms present on affected trees, such as *Hypoxyylon* spp. and insects that attack weakened trees, had confounded our initial abilities to accurately diagnose this mortality, and for some time the incident was viewed as an example of classic oak decline, but without an adequate explanation for the presence of the advancing front. In addition, the difference in coloration of the phloem lesions between *Q. glaucooides* and the other two oak species precluded us from suspecting a common pathogenic cause. Symptoms caused by *Phytophthora* spp. on many plant species often are mistaken for injury from other pathogens or abiotic factors (11), partially resulting from ineffective

isolation techniques (2), and have caused these important plant pathogens to be ignored or overlooked in their role in tree declines and mortality.

P. cinnamomi parasitizes more than 1,000 plant species, many of which are trees (2). It causes the destructive jarrah decline in Western Australia (6,9) and threatens destruction of the ancient, species-rich heath communities on that continent (13). It recently has been associated with decline of *Q. ilex* and *Q. suber* in southern Spain and Portugal (1,12), *Quercus* spp. in California (5), and is perhaps also involved with similar declines of *Quercus* spp. in Italy, Morocco, and Tunisia (1). In southwestern France, the introduced *P. cinnamomi* causes a multiple basal canker on trunks of the also introduced *Q. rubra* (7) but is not generally associated with premature tree death there.

The second reason why this research is significant is that it describes the first 10+ years of an epiphytotic caused by *P. cinnamomi* in a susceptible oak forest shortly after the epiphytotic began. This is the first documentation of the early stages of an epiphytotic following the introduction of *P. cinnamomi* into what is apparently a forest of few but very susceptible species. The exact time and place of introduction of *P. cinnamomi* into El Arrayanal may never be known for sure, although discussions with the villagers suggested that it may have been transported into the village on mango (*Mangifera indica*) plants that were transplanted at one of the residences. Experimental evidence (F. H. Tainter, *unpublished*) suggests that the understory shrub *B. crassifolia* is also susceptible to infection by *P. cinnamomi*, and its early disappearance, as noted by the villagers, may have been a forewarning of imminent oak mortality.

Observations of phloem canker development on inoculated trees in the field trials, and on naturally infected trees, indicate that the apparently susceptible *Q. glaucooides* exhibits no callus response to infection; whereas *Q. peduncularis*, and especially *Q. salicifolia*, do exhibit vigorous callus growth around phloem cankers, which may account for their apparent greater tolerance to infection.

The affected area in the ejido is still small enough so that control efforts have a reasonable chance of being successful and could well serve as a resource for demonstrations, not only for diagnosing the presence of *P. cinnamomi* in soil and symptomatic trees, but also for assessing efforts for its control. There are reports of oak

decline and mortality in other Mexican states. Are these also caused by *P. cinnamomi*? Once the investigator knows what to look for, such cases should be relatively easy to diagnose.

ACKNOWLEDGMENTS

We thank Steve Jeffers, Clemson University, for confirming the identity of the Mexican isolate of *P. cinnamomi*, and Florence Waller, Institut National Agronomique Paris-Grignon, Paris, France, for assisting with soil dilutions and identifying the *Pythium* groups. This research was partially supported by the McIntire-Stennis Cooperative Forestry Program. Eugene Van Arsdel, Tijeras, NM, provided supplementary funding.

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