

Sudden Oak Death and Associated Diseases Caused by *Phytophthora ramorum*

J. M. Davidson, Pacific Southwest Research Station, USDA Forest Service, P.O. Box 245, Berkeley, CA 94701, and Department of Plant Pathology, One Shields Ave., University of California, Davis 95616; **S. Werres**, Biological Research Centre for Agriculture and Forestry, Institute for Plant Protection in Horticulture, Messeweg 11/12, D-38104 Braunschweig, Germany; **M. Garbelotto** Department of Environmental Science, Policy and Management, Ecosystem Science Division, 151 Hilgard Hall, University of California, Berkeley 94720; **E. M. Hansen** Department of Botany and Plant Pathology, Oregon State University, Corvallis 97331; **D. M. Rizzo** Department of Plant Pathology, One Shields Ave., University of California, Davis 95616

Corresponding author: J. M. Davidson. jmdavidson@ucdavis.edu

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Disease

On tanoak (*Lithocarpus densiflora*) and some oak species (*Quercus* spp.): Sudden Oak Death. On other hosts: Ramorum leaf blight and ramorum shoot dieback.

Pathogen

Phytophthora ramorum (S. Werres, A. W. A. M. de Cock & W. A. Man in't Veld) (24).

Taxonomy

Phytophthora ramorum was first described as a pathogen of ornamental rhododendron (*Rhododendron*) and viburnum (*Viburnum*) in Germany and The Netherlands (24). The morphology and ITS sequence of California and Oregon isolates are identical to European isolates (9,19).

Host Range

To date, 23 species in 12 plant families have been found to be naturally infected by *Phytophthora ramorum* (Table 1) (18,19,24). Species in the *Ericaceae* and *Fagaceae* appear to be especially prone to infection. Many more plant species are susceptible to leaf blight in artificial inoculation trials.

Table 1. Known hosts, plant part infected, and potential impact of *Phytophthora ramorum*.

Host species ¹	Family	Common name	Location ²	Plant part infected and impact
<i>Acer macrophyllum</i>	Aceraceae	Big leaf maple	CA	foliar lesions; long term impact unknown
<i>Toxicodendron diversilobum</i>	Anacardiaceae	Poison oak	CA, OR	Limited stem canker; long term impact unknown
<i>Lonicera hispidula</i>	Caprifoliaceae	Honeysuckle	CA	foliar lesions; long term impact unknown
<i>Viburnum</i> spp. ³	Caprifoliaceae	Viburnum	CA, OR, E	Stem lesions
<i>Arbutus menziesii</i>	Ericaceae	Madrone	CA	branch cankers, foliar lesions; death of regeneration and possibly large trees
<i>Arctostaphylos manzanita</i>	Ericaceae	Manzanita	CA	stem and branch cankers, foliar lesions; dieback of branches
<i>Kalmia latifolia</i>	Ericaceae	Mountain laurel	E	foliar lesions; long term impact unknown
<i>Pieris</i> spp. ⁴	Ericaceae	Andromeda	CA, OR, E	stem cankers and foliar lesions; long term impact unknown
<i>Rhododendron</i> spp. ⁵	Ericaceae	Ornamental rhododendron	CA, OR, E	stem and branch cankers, foliar lesions; dieback and death of plants
<i>Vaccinium ovatum</i>	Ericaceae	Evergreen huckleberry	CA, OR	stem and branch cankers, foliar lesions; dieback of canes and possible death of plants
<i>Lithocarpus densiflora</i>	Fagaceae	Tanoak	CA, OR	stem and branch cankers, foliar lesions; death of large trees, saplings and regeneration
<i>Q. chrysolepsis</i>	Fagaceae	Canyon live oak	CA	Stem cankers, death of saplings, possible death of large trees
<i>Q. kelloggii</i>	Fagaceae	California Black oak	CA	stem cankers; death of large trees

Table 1 (continued). Known hosts, plant part infected, and potential impact of *Phytophthora ramorum*.

Host species ¹	Family	Common name	Location ²	Plant part infected and impact
<i>Q. parvula</i> var. <i>shrevei</i>	Fagaceae	Shreve's oak	CA	stem cankers; death of large trees
<i>Quercus agrifolia</i>	Fagaceae	Coast live oak	CA	stem cankers; death of large trees
<i>Aesculus californica</i>	Hippocastanaceae	California buckeye	CA	foliar lesions; long term impact unknown
<i>Umbellularia californica</i>	Lauraceae	Bay laurel, Oregon myrtle	CA	foliar lesions; long term impact unknown
<i>Pseudotsuga menziesii</i>	Pinaceae	Douglas-fir	CA	branch cankers, foliar lesions; death of new shoots and small branches
<i>Rhamnus californica</i>	Rhamnaceae	Coffeeberry	CA	foliar lesions; long term impact unknown
<i>Rhamnus purshiana</i>	Rhamnaceae	Cascara	OR	foliar lesions; long term impact unknown
<i>Heteromeles arbutifolia</i>	Rosaceae	Toyon	CA	branch cankers, foliar lesions; branch dieback, long term impact unknown
<i>Rubus spectabilis</i>	Rosaceae	Salmon berry	OR	Foliar lesions; long term impact unknown (Koch's Postulates in progress)
<i>Sequoia sempervirens</i>	Taxodiaceae	Coast redwood	CA	branch cankers, foliar lesions; death of sprouts, decline of saplings
<i>Camellia</i> spp. ⁶	Theaceae	Camellia	CA, E	Leaf lesions; long term impact unknown

1 Cultures of *P. ramorum* have been obtained from all hosts with the exception of *A. manzanita*.

2 Location from which *P. ramorum* has been observed on named host to date. CA, California; OR, Oregon; E, Europe.

3 Viburnum species from which *P. ramorum* has been recovered include: *V. bodnantense*, *V. fragans*, *V. plicatum*, and *V. tinus*.

4 Pieris species and cultivars infected by *P. ramorum* include: *P. formosa* var. *forrestii* and *P. japonica*.

5 Rhododendron species and cultivars from which *P. ramorum* has been isolated include: *Rhododendron catawbiense*, *R. macrophyllum*, Catawbiense Grandiflorum, Catawbiense Boursalt, Schneewolk, Colonel Coen, Gomer Waterer, and Cunningham's White.

6 Camellia species and cultivars from which *P. ramorum* has been isolated include: *Camellia japonica* (Kramer's Supreme, Kumasaka, Mrs. Charles Cobb, and Silver Waves); and *Camellia sasanqua* (Bonanza).

Geographical Distribution

The current known distribution of *P. ramorum* includes the western USA, Europe, and Canada. In the USA, the distribution of *P. ramorum* within natural areas is patchy, with sites extending in a narrow coastal band over 600 km from Monterey County, CA (36°15'N, 121°47'W) to Curry County, OR (42°6'N, 124°15'W) (9,19). The wildland sites currently furthest inland in California are approximately 70 km from the coast in Solano County (38°18'N, 122°12'W). Although these sites include all known hosts, geographic range surveys have thus far used dead oak and tanoak as indicators for disease, and therefore may underestimate the true range by failing to identify areas in which *P. ramorum* is only present on non-oak hosts. In addition to these wildland sites, *P. ramorum* has been found on rhododendron, camellia (*Camellia*), viburnum, and andromeda (*Pieris*) in nurseries in California, Oregon, Washington, and British Columbia that are within and outside the range of *P. ramorum* in natural areas (C. Blomquist, *unpublished data*; E. Hansen, *unpublished data*; G. Kristjansson, *unpublished data*; N. Osterbauer, *unpublished data*; A. Wagner, *unpublished data*). For the most recent information on the North American geographical distribution of *P. ramorum*, see www.suddenoakdeath.org.

In Europe, *P. ramorum* has been isolated from symptomatic rhododendron and viburnum in nurseries, gardens and landscape plantings in Germany, the Netherlands, the United Kingdom, Poland, Spain, France, Belgium, and Sweden (6,7,12,15,17,23,24). *P. ramorum* has also been found to infect camellia, andromeda (*Pieris* spp.), and mountain laurel (*Kalmia latifolia*) in European nurseries (11; P. Beales, *unpublished data*; T. Brokenshire, *unpublished data*). Up to now, *P. ramorum* has not been recovered from plants in forests in Europe.

Symptoms and Signs

Oaks (*Quercus* spp.). To date, only oaks in the red oak group (section *Lobatae*) have been found to be susceptible to infection by *P. ramorum* in California (19). These include coast live oak (*Quercus agrifolia*), California black oak (*Quercus kelloggii*), and Shreve's oak (*Quercus parvula* var. *shrevei*). The remaining species in the red oak group in California, interior live oak (*Q. wislizenii*), has been found to be susceptible to infection in greenhouse inoculation trials (D. M. Rizzo, *unpublished data*). In addition, northern red oak (*Quercus rubra*) and pinoak (*Q. palustris*), important species from eastern North America, have been shown to be susceptible in greenhouse inoculation studies (18; Hansen, *unpublished data*). Canyon live oak (*Q. chrysolepsis*) in the golden cup group (section *Protobalanus*) has been found infected by *P. ramorum* at a single location (16). No species in the white oak group (section *Quercus*) have been found with the disease in the field in California, Oregon, or Europe.

In oaks, only adult plants are significantly affected. Infection of seedlings is unreported in nature, and infection of saplings appears to be extremely rare. The most useful diagnostic symptom for *P. ramorum* is the development of cankers on the trunk. Cankers have red-brown to black discoloration and seep dark black to red or amber sap (Figs. 1A and 1B). They usually develop 1 to 2 m off of the ground, although they can be at soil level, or as high as 4 m or greater. Evidence to date suggests that cankers do not extend into the roots below the soil line. Cankers will occasionally extend onto portions of buttress roots if the roots are above the soil line.

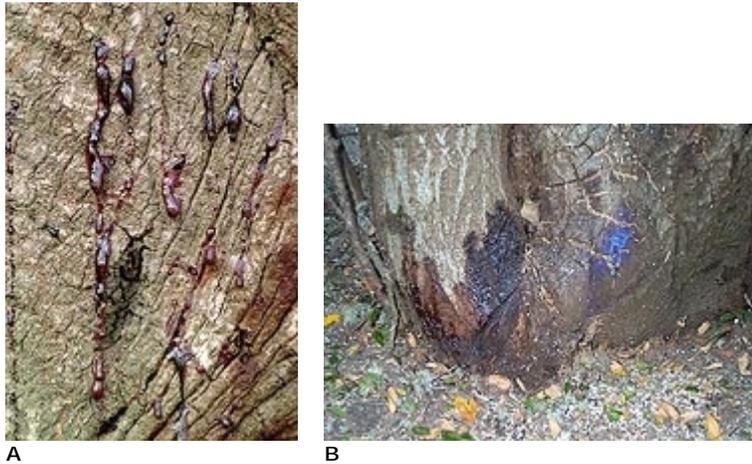


Fig. 1. *P. ramorum* canker on coast live oak. Outer bark shows rust-colored seepage (A, B).

Typically, canker formation begins with isolated bleeding spots on the main trunk of the tree. At the early stages of canker development, seeping will occur through the intact bark, without any noticeable physical wounding. In later stages, the bark can fracture and exudation occurs both through broken and intact bark. Later, the area will be stained reddish-brown. When liquid, the sap smells like the oak tannin odor of wine barrels. Dark wet cankers on oak trunks with very foul smelling exudates are usually due to bacterial infections (wetwood), not *P. ramorum*. The sap from a *P. ramorum* canker may dry and harden into a blackish crust during the summer months. Infection and discoloration is generally more extensive in bark and phloem tissues than in the xylem. In active cankers, the phloem is discolored various shades of brown (Fig. 2A), and cankers are delimited by thin black lines (Fig. 2B). Cankers may be entire, or consist of smaller separate cankers in close proximity. It is unknown at this point whether these represent separate infections or just an uneven spread of the pathogen. Black discoloration can extend to 3 cm or greater into the xylem below the bleeding canker, but more typically extends less than 1 mm. Cankers may range in size from a few cm in diameter to 3 m in length. *Phytophthora* is typically isolated from the margins of these discolored areas, although it can occasionally be isolated from older portions of the cankers.

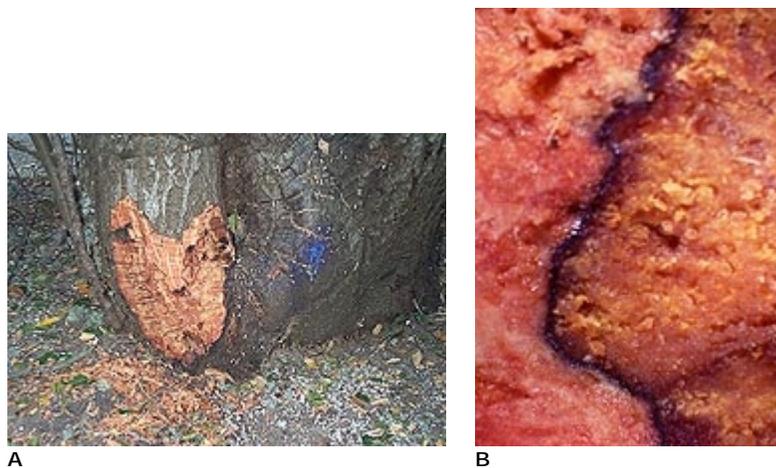


Fig 2. In coast live oak, removing outer bark reveals diseased phloem tissue and zone lines (A). Thin black zone lines characteristically separate diseased from healthy tissue in oak and tanoak cankers (B).

Phytophthora ramorum canker disease of oak has been called “Sudden Oak Death” due to the rapid (2 to 4 weeks) and complete browning of the crown observed on numerous trees at their death (Fig. 3). In these cases, the dead foliage appears without a prolonged period of visible decline. While this sudden browning may occur, death of the tree due to *P. ramorum* infection usually takes place after an extended period of disease and perhaps more than two years from

the onset of infection. The crown of the tree can appear healthy in early stages of the disease, although a bleeding canker is present on the trunk. The browning may occur several months to over a year after the pathogen has effectively girdled the whole stem. Some infected trees may experience gradual leaf loss and death rather than a sudden browning of the crown.



Fig. 3. Several years after onset of infection, rapid browning and death of all leaves occurs in some oak trees infected with *P. ramorum* canker disease.

Other *Phytophthora* species can cause canker symptoms on oak identical to those of *P. ramorum*. In urban areas of California, *P. cinnamomi*, *P. citricola*, and *P. cactorum* have been isolated from bleeding cankers on oak trees in irrigated lawns (14). *P. cinnamomi* has been shown to cause similar symptoms on several oak species in natural forests in Mexico (21). In Europe, *P. cambivora*, *P. cinnamomi*, and other *Phytophthora* species have been isolated from bleeding cankers often described as “ink disease” (1). These all differ in having a root origin, although this is sometimes difficult to determine. While *P. ramorum* may be distinguished from these other *Phytophthora* species by its apparently exclusive aerial biology, pathogen isolation is needed to conclusively show that canker symptoms are caused by *P. ramorum* and not another *Phytophthora* species. In California and Oregon forests, an as yet unnamed *Phytophthora* species, closely related to *P. ilicis*, causes very similar canker symptoms on oaks (3,19).

A number of opportunistic organisms are commonly observed on oak trees with advanced *P. ramorum* infections. These include ambrosia beetles (*Monarthrum scutellare* and *M. dentiger*), bark beetles (*Pseudopityophthorus pubipennis*), and a sapwood rotting xylariaceous ascomycete, *Hypoxylon thouarsianum*.

Tanoak (*Lithocarpus densiflora*). Tanoak is the most susceptible of the known hosts to *P. ramorum*. Non-wound inoculation experiments indicate that *P. ramorum* can kill tanoaks within one year from onset of infection (J. M. Davidson, *unpublished data*). Increasing numbers of sites in California have dramatic mortality of tanoaks (e.g., Muir Woods National Monument, Pfeiffer Big Sur State Park, Marin Municipal Water District). All size classes (seedlings, saplings, mature trees) of tanoak are infected and can be killed by the disease.

Saplings: *P. ramorum* causes cankers on trunks, branches, and twigs; and lesions on leaves and petioles of saplings. Trunk and branch cankers are discolored areas, slightly darker than the grey bark. They do not bleed. Scraping the outer bark of a canker reveals brown or tan diseased tissue with zone lines (Figs. 4A and 4B). Cankers on twigs and small branches may or may not be sunken (Figs. 5 and 6). *P. ramorum* infection in the terminal twigs can lead to shoot tip dieback, leaf flagging (Fig. 6), or the formation of a Shepard’s crook (Fig. 7). In laboratory inoculation experiments, infections spread within a leaf and from twigs into leaves. Other pathogens, such as *Diplodia* have also been isolated from twig dieback and twig cankers on tanoak (S. T. Koike, *unpublished data*).

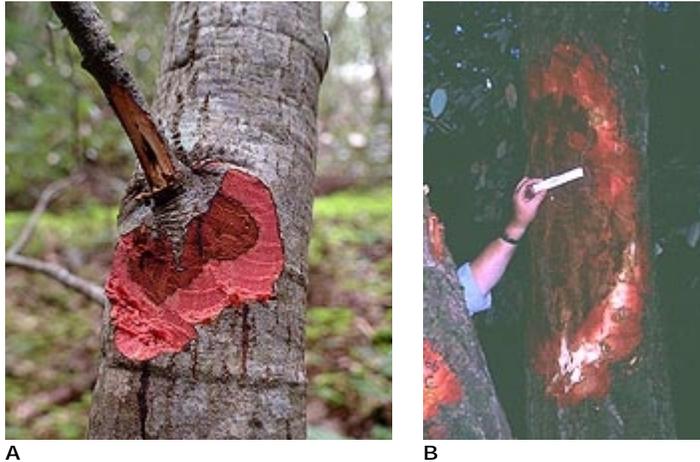


Fig. 4. *P. ramorum* canker on tanoak adult from natural (A) and artificial (B) inoculation.



Fig. 5. *P. ramorum* canker on tanoak terminal twig.



Fig. 6. *P. ramorum* stem canker and corresponding leaf flagging on tanoak sapling.



Fig. 7. Tanoak sapling with shepherd's crook due to *P. ramorum* infection.

Mature trees: *P. ramorum* also infects trunks, branches, twigs, leaves, and leaf petioles of adult trees. Trunk cankers are similar to those of the red oak group (Figs. 4A and 4B). Cankers have been observed as high as 25 m on tanoak. Wound and non-wound inoculation experiments on adult trees revealed that trees could be infected but not show cankers or bleeding symptoms on the outer bark, making diagnosis of tanoak difficult (19; Davidson, *unpublished data*). Active bark cankers may have a zone of water-soaked tissue around the more visible necrotic area. Symptoms on branches, twigs, and leaves are as stated for tanoak saplings. As with the red oaks, death can occur with a sudden browning of the leaves, or over time with gradual leaf loss. Leaf flagging is a particularly good indicator of disease (Fig. 6). As the above-ground parts of the tree die, it may resprout from the base of the trunk. However, sprouts generally die with the tree. These shoots, as well as stump sprouts, are very susceptible while succulent. As in diseased mature oaks, a similar array of opportunistic organisms is associated with dying tanoaks.

Rhododendron (*Rhododendron* spp.). *P. ramorum* causes leaf lesions and branch dieback in several rhododendron species. Stems with stem blight develop a brown to black discoloration (Figs. 8A and 8B). Usually the discoloration starts at the tip of the twig and moves towards the base. However,

the disease can also start at any other part of the stem and move upwards, downwards, or into the leaves. The cambial tissue of the infected stem may be discolored and the wood can also turn brown (Fig. 8C). Stem tissue may be killed. Dieback of wild rhododendron may extend into the main stems and result in plant death. Leaf infection can occur with and without prior twig infection. In leaves infected from a twig infection via the petiole, necrosis is often concentrated along the prominent midvein (Fig. 9A). Leaves infected without prior twig infection can show distinct leaf spots on any part of the leaf surface (Figs. 9B to 9D). Roots do not seem to become infected, but *P. ramorum* may still be present in the soil.

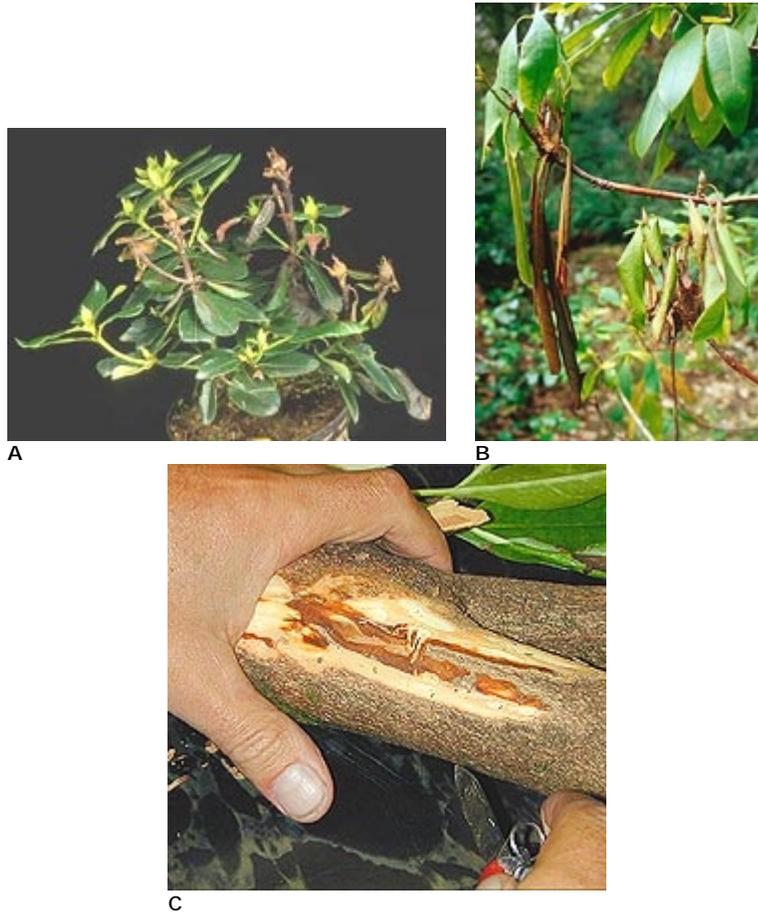


Fig. 8. Stem dieback in rhododendron due to *P. ramorum* (A and B). Discoloration can occur in the infected cambium (C).



Fig. 9. Infected rhododendron leaves have dark brown lesions. Necrosis is often concentrated along the midvein in leaves infected from a twig infection via the petiole (A). Leaves infected without prior twig infection can have leaf spots on any part of the leaf surface (B, C, and D). 9D shows naturally infected leaves of the native rhododendron, *R. macrophyllum*, from southwestern Oregon.

Initially, symptoms of *P. ramorum* on rhododendron and/or azalea can be very much like those of other *Phytophthora* species such as *P. cactorum*, *P. citricola*, *P. cinnamomi*, and *P. syringae*. There are other damaging agents that may cause twig or stem dieback (e.g., *Phomopsis* sp., *Botryosphaeria* sp.) or wilting symptoms (e.g., *Cylindrocladium* sp., root weevils, drought, or water stress). Leaf lesions caused by *P. ramorum* can sometimes be confused with early symptoms of rust infection prior to the development of visible spore forms of either pathogen on the undersides of the leaves. In addition, it may be difficult to distinguish between leafspots caused by *P. ramorum* and those caused by sunburn. However, the margin on *Phytophthora* lesions tends to be diffuse, while the margin on sunburn is distinct. The extension of necrosis due to *P. ramorum* along the midvein also is often distinctive.

In Oregon and California, *P. ramorum* infects native *R. macrophyllum* (Fig. 9D) growing among infected tanoaks (9). Ornamental rhododendrons in California and Europe also have been found infected with *P. ramorum*. Susceptible nursery varieties include, but are not limited to: Catawbiense Grandiflorum, Catawbiense Boursalt, Schneewolk, Colonel Coen, Gomer Waterer, and Cunningham's White. *Rhododendron catawbiense* has also been found to be susceptible to infection in German nurseries, but *P. ramorum* does not seem to damage *Rhododendron simsii* in nurseries. Experiments are underway to investigate the susceptibility of azaleas. Initial inoculation studies under controlled conditions indicate that some azaleas (e.g., Northern Highlights and *R. occidentale*) are susceptible to *P. ramorum*. Deciduous azaleas generally seem to be more susceptible than evergreen azaleas (22).

Madrone (*Arbutus menziesii*). Symptoms and disease progression on madrone are similar to that seen on rhododendron with leaf spots, leaf death, and branch dieback commonly associated with infection by *P. ramorum*. *P. ramorum* forms very dark lesions on leaves (Fig. 10), often extending down petioles and into the twig. On stems, the lesions initially are difficult to find and appear as a slight water soaking or darkening of the bark that travels very quickly throughout the stem and kills the tissue, creating a dark lesion (Fig. 11)

(P. E. Maloney, *unpublished data*). Necrosis may progress from infected stems into leaves.



Fig. 10. Leaf and stem lesions on madrone sapling due to *P. ramorum*. The sapling was placed in an infested forest to ensure a natural infection process.



Fig. 11. Close-up of *P. ramorum* stem canker on madrone in Figure 10.

P. ramorum has been confirmed from leaf lesions and stem cankers on madrone and manzanita (*Arctostaphylos manzanita*) using molecular, and to a lesser extent, cultural techniques. Difficulty in culturing *P. ramorum* from lesions, in addition to lack of unique symptomatology, complicates diagnosis of *P. ramorum* infection of madrone. Presently, molecular PCR-based techniques are the best way to confirm presence of *P. ramorum* in madrone.

From Northern California to British Columbia, madrone is infected with *Nattrassia mangiferae* and *Botryosphaeria dothidea* (8). These pathogens overlap in geographic range with *P. ramorum*. *N. mangiferae* causes distinct sunken cankers that look like open sores on the branches and trunk. *B. dothidea* causes dieback of twigs and leaves that could be confused with symptoms of infection by *P. ramorum*. Generally, neither pathogen causes death of madrone.

Evergreen huckleberry (*Vaccinium ovatum*). The most distinctive symptoms of *P. ramorum* on huckleberry are small cankers on the stems (Fig. 12) and cane dieback. Cankers on the stems are dark and tend to be confined to the outer bark. On larger stems with dieback, the use of a knife to peel outer bark back from the edge of a canker will often reveal yellow discoloration in the pith. Observations of huckleberry plants in areas of *P. ramorum* infection suggest that stem dieback may kill the plant, although disease progression has not been extensively followed. Laboratory inoculation experiments show that *P. ramorum* can infect and cause lesions on leaves. However, *P. ramorum* has not been isolated from leaf spots in the field, perhaps because infected leaves often abscise quickly after infection. Although entire plants can be killed, it is more common to observe patches of dead and live branches.



Fig. 12. Stem canker on huckleberry due to *P. ramorum*.



Fig. 13. Leaf spots and stem dieback on manzanita inoculated with *P. ramorum*.

Manzanita (*Arctostaphylos manzanita*).

Symptoms and disease progression have not been well characterized on this host. *P. ramorum* has been identified from stem cankers in the field using molecular techniques. Infection with reisolation has been demonstrated in the lab, but there has been no isolation from field material. Based on field observations and inoculations, branch symptoms appear to be similar to that seen with madrone. Lesions appear as a darkening of the bark that travels very quickly throughout the stem and kills the tissue (Fig. 13). Necrosis may progress from infected stems into leaves.

Bay laurel (Oregon myrtle) (*Umbellularia californica*). *Phytophthora ramorum* causes leaf lesions on bay laurel (Fig. 14). Lesions are brown and often, but not always, are delimited by a chlorotic (i.e., yellow) zone. Lesions typically occur where water collects on the leaf, generally on the leaf tip. On leaves that are flat or carried with the tip upwards, a lesion

can develop in the middle section, especially around the edges, or at the base of the petiole. These types of leaf spots can also be caused by anthracnose (*Colletotrichum gloeosporioides*), therefore pathogen isolation is necessary to verify the presence of *P. ramorum*. An as yet unnamed *Phytophthora* species, closely related to *P. ilicis*, is commonly isolated from bay leaves in tanoak-redwood forests in California and Oregon and causes identical symptoms (3). Some branch dieback has been observed in trees infected by *P. ramorum*, but it is still uncertain whether *P. ramorum* is responsible for the development of this symptom because the pathogen has not been recovered from dying branches. Mortality of bay laurel due to infection by *P. ramorum* has never been reported. Bay trees are thought to be important reservoirs of inoculum because the pathogen readily sporulates on water-moistened bay leaves (5).



Fig. 14. *P. ramorum* lesion on leaf tips of bay laurel.

California buckeye (*Aesculus californica*). *P. ramorum* causes light brown leaf spots on buckeye (Fig. 15A). These spots are often not round, but may form a square necrotic region bounded by leaf veins. *P. ramorum* can infect the leaves, the petioles, and the twigs of California buckeye trees. Early symptoms start as rounded individual spots that tend to coalesce later in the season (Fig. 15B). The symptoms are very similar to those caused by the buckeye anthracnose pathogen, *Guignardia aesculi*. Darkened lesions on petioles and twigs (Fig. 16) indicate presence of *P. ramorum*. Early foliar senescence (buckeyes are deciduous trees and drop the foliage unusually early [June] in hot and dry areas) causes leaf distortions similar to those caused by *P. ramorum*.



A
B
 Fig. 15. Intervein leaf lesions (A) on buckeye due to *P. ramorum* often coalesce (B).



Fig. 16. Stem canker on buckeye.

Big leaf maple (*Acer macrophyllum*). In this tree species, *P. ramorum* infection appears more like a scorch, normally starting from the edges of the leaf. In general, the scorching will have irregular borders that do not precisely follow leaf contour. The discoloration can be variable in color with hues ranging from orange to brown (Figs. 17A and 17B). In artificial inoculations, very dark necrotic lesions are formed, often at the base or the tip of the leaf blade where water accumulates. Once naturally infected leaves senesce and yellow, the discoloration is often still visible for some time. Branch die-back has been observed in areas infested by *P. ramorum*, but a clear cause-and-effect relationship has not been determined yet.

Other disorders with similar symptoms include drought stress, *Xylella* leaf scorch, and anthracnose (e.g., *Discula* sp.) (20).



A B
Fig. 17. Symptoms of *P. ramorum* infection in leaf of big leaf maple (A, B).

Toyon (*Heteromeles arbutifolia*), coffeeberry (*Rhamnus californica*), cascara (*Rhamnus purshiana*), salmon berry (*Rubus spectabilis*), and honeysuckle (*Lonicera hispidula*). Disease symptoms have not been well characterized on these hosts at this time. Leaf lesions are characteristically round with a bulls-eye appearance of alternating light and dark rings (Figs. 18 to 21). On salmon berry leaf, lesions have a water-soaked appearance. Lesions may appear on the main part of the leaf or leaf tip. *P. ramorum* may also cause stem dieback on toyon and dieback of basal sprouts on cascara. (Note that Koch's postulates have been completed for all hosts listed except salmon berry, where they are in progress.)



Fig. 18. *P. ramorum* leaf lesion on toyon.



Fig. 19. *P. ramorum* leaf lesion on coffeeberry.



Fig. 20. *P. ramorum* leaf lesion on cascara.

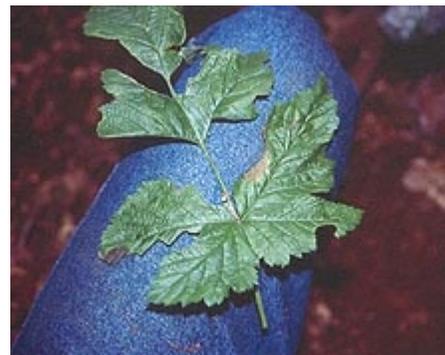


Fig. 21. *P. ramorum* lesions on salmon berry leaf tips.

Poison oak (*Toxicodendron diversilobum*). Field symptoms observed to date are limited to bleeding stem cankers on poison oak stems climbing around infected tanoak trees (Figs. 22A and 22B). In artificial inoculations, water-soaked lesions on leaves are also formed after dip inoculation.



A
B
Fig. 22. Bleeding canker on poison oak stem due to *P. ramorum* (A). B shows cambial tissue under canker.

Viburnum (*Viburnum* spp.). Viburnum species known to be infected by *P. ramorum* include *V. bodnantense*, *V. fragans*, *V. plicatum*, and *V. tinus* (12,23). Infected viburnum show wilting symptoms (Fig. 23). At later stages, single twigs or the whole plant collapses. Collapse can occur within several days of initial infection. In contrast to the disease on rhododendron, twig blight or dieback has not been observed in viburnum. Instead, *P. ramorum* spreads from the stem base upwards. Due to the bark structure, symptoms on the outer bark, such as discoloration, are very difficult to distinguish. However, bleeding occasionally occurs on infected viburnum stems (Fig. 24). Under the outer bark, necrotic cambial tissue is discolored (Fig. 25) and looks similar to cambium cankers on trees attacked by *Phytophthora* spp. The roots of the attacked viburnum usually appear healthy. Wilting of viburnum caused by *P. ramorum* can be confused with numerous other damaging agents such as *Verticillium*, drought, or water stress.



Fig. 23. Viburnum infected with *P. ramorum* showing wilting symptoms.



Fig. 24. *P. ramorum* infection occasionally causes bleeding on viburnum stems.



Fig. 25. Discoloration indicates cambial canker in viburnum stem due to *P. ramorum* infection.

Douglas-fir (*Pseudotsuga menziesii*). On Douglas-fir saplings, *P. ramorum* causes cankers on small branches that result in wilting of new shoots, dieback of branches, and loss of needles (Fig. 26). On small saplings, (<1 m in height), infection by *P. ramorum* can kill the leader shoot and top several whorls of branches (Fig. 27). To date, disease symptoms have not been observed on adult trees (4). Studies are underway to characterize disease progression on Douglas-fir and determine whether terminal branch infections lead to decreased growth or main trunk infections.



Fig. 26. Dieback of Douglas-fir shoots due to *P. ramorum*.



Fig. 27. Leader shoot and top whorls of branches on Douglas-fir sapling killed by *P. ramorum*.

Redwood (*Sequoia sempervirens*). *P. ramorum* causes leaf lesions, cankers on small branches, and dieback of epicormic sprouts on redwood saplings and adults. Leaf lesions may have a darkened zone line between necrotic and live tissue (Fig. 28). Cankers on branches may be noticeable due to browning and needle loss at the tip or middle of a branch (Fig. 29). Dieback of stump sprouts results in blackened, withered tips that become brown as they dry out (Fig. 30). The effect of infection on redwood currently is unknown. However, *P. ramorum* appears to kill sprouts and cause loss of vigor in saplings. To date, disease symptoms have not been observed on adult tree trunks (13).



Fig. 28. Leaf lesions on redwood leaves.



Fig. 29. *P. ramorum* canker on redwood twig and corresponding needle loss.



Fig. 30. Dieback of redwood epicormic stump sprouts due to *P. ramorum*.

Camellia (*Camellia* spp.), andromeda (*Pieris* spp.), and mountain laurel (*Kalmia latifolia*). *P. ramorum* causes leaf lesions on all of these species (Figs. 31A to 31D, 32A, and 33), as well as stem lesions on andromeda (Fig. 32B) (11; P. Beales, *unpublished data*; C. Blomquist, *unpublished data*; T. Brokenshire, *unpublished data*). Leaf lesions on camellia may spread to the entire leaf, and infected leaves tend to abscise. Susceptible species and varieties of *Camellia* include: *C. japonica* (Kramer's Supreme, Kumasaka, Mrs. Charles Cobb, and Silver Waves); and *C. sasanqua* (Bonanza). Susceptible species and varieties of *Pieris* include *P. formosa* var. *forrestii* and *P. japonica*. Koch's postulates have been completed for *C. japonica*, *P. formosa* var. *forrestii*, *P. japonica*, and *Kalmia latifolia* (11; P. Beales, *unpublished data*).



Fig. 31. Leaf lesions due to *P. ramorum* on *Camellia japonica* varieties Kramer's Supreme (A), Mrs. Charles Cobb (B), Silver Waves (C), and Kumasaka (D). (Courtesy C. Blomquist, California Department of Food and Agriculture.)

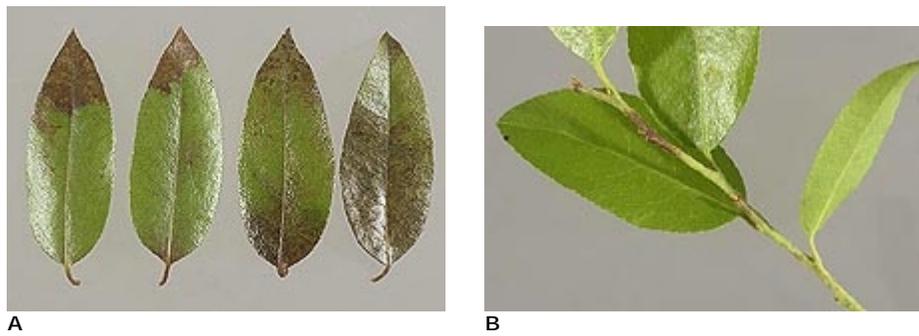


Fig. 32. Leaf lesions (A) and stem lesions (B) on andromeda caused by *P. ramorum*. (Courtesy S. Ashby, Department for Environment, Food and Rural Affairs, United Kingdom.)



Fig. 33. Leaf lesions on *K. latifolia* due to *P. ramorum*. (Courtesy S. Ashby, Department for Environment, Food and Rural Affairs, United Kingdom.)

Pathogen Isolation

Phytophthora ramorum can be isolated from infected plant material using standard practices for other *Phytophthora* species such as pimaricin-ampicillin-rifampicin-PCNB (PARP) selective medium (19), PARP without the PCNB (CARP) (10), and carrot piece agar (24). For all isolations, plated material is taken from the leading edge of a canker or lesion. Ingredients such as fungicides and antibiotics in the selective media may sometimes suppress the outgrowing of hyphae from plant tissue and/or the development of *Phytophthora* propagules.

Isolation from cankers on oak or tanoak is best done in the field. Less material needs to be taken from the tree and the sample does not dry out. The best place to sample is from an area with active bleeding on the outer bark because it increases the chances that the inner bark, or phloem, will be moist and the infection fresh. After shaving away outer bark, identify the canker and surrounding zones lines separating diseased and healthy tissue. Diseased tissue can be the same color as healthy inner bark, tan, or dark brown. Use a scalpel to remove 12 to 15 approximately 2 mm² pieces of bark that span the zone line and embed these in PARP. Incubate plates at 18°C in the dark. Mycelia of *P. ramorum* from positive cultures are usually visible within four to six days, although it is worth keeping plates for three weeks to score for any late-emerging isolates. Potential positive isolates should be transferred to new plates for further identification.

Obtaining positive samples from woody tissue can be difficult. Success rate from positive-appearing trunk cankers on oaks and tanoaks in sites known to have *P. ramorum* disease is approximately 55%. The more pieces of tissue plated, the greater the probability of a positive sample. It may also prove useful to take samples from several different patches on the canker. In California, isolation success appears to decline in the late summer months for most hosts.

For foliage and branch isolations, small pieces of tissue from the leading edge of a lesion or canker are plated onto a selective medium, and the isolation procedure follows the protocol outlined above. Studies on isolations from bay laurel leaves reveal an effect of seasonality: isolation success declines throughout the dry summer months from approximately 90% in June to 25% in October (J. M. Davidson, *unpublished data*). Pathogen isolation from other host species, including oaks, also shows this pattern. Therefore, in dry seasons, a greater number of leaves or bark pieces may need to be sampled to compensate for lowered isolation success. Although data are limited, isolation from Douglas-fir terminal branches and coffeeberry leaves is successful in the spring with recent infections, while we fail to isolate *P. ramorum* from these hosts at other times of the year. With other host plant species such as madrone, big leaf maple, toyon, buckeye, and honeysuckle, isolation is difficult and we tend to rely on molecular amplification of *P. ramorum* DNA to verify its presence. For those species from which *P. ramorum* is rarely recovered by traditional culturing methods, but is shown to be present by molecular diagnostics, it is unclear whether *P. ramorum* is killed by plant defenses, out-competed by other pathogens, or no longer viable after successfully destroying the tissue of its plant host.

Diagnosis Via PCR

DNA for PCR-based detection can be extracted directly from plant tissue; alternatively, PCR can be used on cultures to confirm their identity. While many different protocols can be adopted for DNA extraction, we describe one that can be applied to all types of plant material, including wood. Symptomatic leaves, twigs, or bark cankers are first frozen and then lyophilized. As in the case of culturing, plant tissue at the margins of the infection should be selected for the extraction. Approximately 10 to 100 mg of plant tissue are placed in an 1.5-ml microfuge tube and ground into a fine powder by placing 2 stainless steel ball-bearings (3/16 inch) in the tube, and shaking it vigorously in an amalgamator for several seconds. The extraction procedure involves hydrating the plant material in a CTAB extraction buffer, using a phenol-chloroform-isoamyl solution to eliminate the proteins, and binding the DNA to silica beads according to manufacturer's instructions for the GeneClean Turbo Nucleic Acid Purification Kit. DNA extracts are then diluted 1:100 to 1:200 in 0.1X TE buffer

before setting up the PCR reaction. In the case of cultures, it is sufficient to place a minute amount of hyphal tissue in a microfuge tube containing 100 to 200 μl of 0.1X TE, vortex it, pellet the cells down by a 2-minute spin in a microfuge, and use the supernatant as template DNA. A single round of 35 cycles is normally sufficient to detect *P. ramorum* DNA in *Rhododendron* spp., and bay laurel leaves. Diagnosis from wood and from other hosts requires more sensitive approaches such as increasing the number of cycles to 40 or a second round of nested PCR using the PCR products from the first round as template.

PCR Reaction

For best detection, two rounds of PCR are necessary. The first round is executed using primers Phyto1 (CATGGCGAGCGCTTGA) and Phyto4 (GAAGCCGCCAACACAAG) and reagents described in Table 2.

Table 2. Recipe of the cocktail for PCR-diagnosis of *P. ramorum*.

Reagent	Stock Conc.	Volume (μl)	Final Conc.
PCR Water	N/A	12.25	N/A
PCR buffer (no Mg)	10X	2.50	1X
DNPTs	2 mM	2.50	0.2 mM
MgCl ₂ *	50 mM	0.75	2.0 mM
Phyto1	50 μM	0.25	0.5 μM
Phyto4	50 μM	0.25	0.5 μM
<i>Taq</i> polymerase	5 U/ μl	0.25	0.05 U/ μl
Template DNA		6.25	
Total Volume		25	

PCR parameters should be as follows:

1 cycle at: 94°C for 1'25"
 34 cycles at: 93°C for 35"
 62°C for 55"
 72°C for 50" adding 5' at each cycle
 1 cycle at: 72°C for 10'
 Ramp rate: 3.3°C/second heating,
 2.0°C/second cooling

The expected PCR product is 687 bp long. The size of the amplicon can be easily verified by agarose gel electrophoresis using the following conditions: 1.5% agarose, 4V/cm, 1 hour run time. For best detection, dilute the PCR product 1:500, and perform a second PCR run with the same parameters described above, but using primers Phyto2 (AAAGCCAAGCCCTGCAC) and Phyto3 (GGTGGATGGGACGTG), instead of Phyto1 and Phyto4. The nested procedure can be four times more sensitive than the single-round approach. Nested PCR products should be 291 bp long. No cross-reactivity with other *Phytophthora* species has been noted with the exception of *P. lateralis* and *P. cambivora*, but only when DNA of these species is present in high concentration. If cross-reactivity with *P. lateralis* and *P. cambivora* is an issue, the problem can be easily eliminated by determining the concentration of template DNA, and by diluting the sample to concentrations at which no cross-reactivity occurs. This diagnostic protocol will only detect *P. lateralis* and *P. cambivora* at concentrations of DNA ≥ 96 pg/ μl and ≥ 56 pg/ μl , respectively. These concentrations of pathogen DNA are rather unlikely to occur in plant tissue. *P. ramorum* DNA, on the other hand, will be detected at concentrations < 0.152 pg/ μl , a 6-order magnitude difference in detection threshold when compared to the other two species. Template DNA can be quantified before PCR in a variety of ways including spectrophotometry, densitometry, and fluorometry. As a general rule, a 1:1000 dilution of a DNA extraction from plant tissue will ensure pathogen DNA concentrations are below the threshold of 56 pg/ μl .

Precautions and controls. At all stages (material preparation, lyophilization, DNA extraction, first PCR round, nested PCR round), negative control samples should be included. Extreme care should be exercised when handling and diluting the first round of PCR as DNA contamination is likely to occur at this stage. A universal primer pair can be added to the traditional PCR reaction as a control on the efficacy of the DNA extraction or PCR reaction (26). Control primers will amplify an amplicon approximately 175 bp in size from all plant and fungal material, and will not interfere with *P. ramorum* primers. Control primers UnivForw (ggaacgtgagctgggttag) and UnivRev (ttctgacttagaggcgttcag) can be added to reach a final 0.5 μ M concentration in the traditional PCR approach. Currently, DNA sequencing is extremely inexpensive and both the 687 bp and the 291 bp amplicons are informative enough to differentiate *P. ramorum* from all other known *Phytophthora* species. We recommend sequencing confirmation for critical samples. Although the diagnostic procedures here described are highly specific for *P. ramorum*, unwanted deviations from the described protocols due to varying quality of reagents and DNA extracts, or to pipette and Thermocycler malfunction, may result in non-specific amplification.

Several other PCR-based procedures based on a range of nuclear and mitochondrial sequences are now available, including Real Time PCR, single strand conformation polymorphism (SSCP) detection, as well as other traditional PCR protocols. The procedure described above in detail can be employed by all laboratories, without the need for sophisticated equipment, and is the only technique based on primers fully matching *P. ramorum* DNA that has been extensively tested with environmental samples.

Pathogen Identification

Phytophthora ramorum has a nearly unique set of morphological characteristics and a unique molecular sequence at the ITS region (24). The most distinguishing feature of *P. ramorum* is the presence of large (22 to 72 μ m) chlamydospores that are mainly terminal (Figs. 34A and 34B). Chlamydospores change from hyaline to cinnamon brown as they mature. This change is especially pronounced in media with host material. Sporangia are semi-papillate, caducous, and will form in clusters on agar with flooding (Fig. 34C). They are highly deciduous in soil water at 20°C. *P. ramorum* has highly branching hyphae on PARP that become septate in older cultures (Fig. 34B). This pathogen appears to be heterothallic. Mating in vitro has been achieved through crosses with other *Phytophthora* species (Fig. 34D) (24). Pairings of European and American isolates in vitro on carrot agar (C. M. Brasier and S. Kirk, *unpublished data*) and in vivo within rhododendron twigs (25) have led to production of oospores.

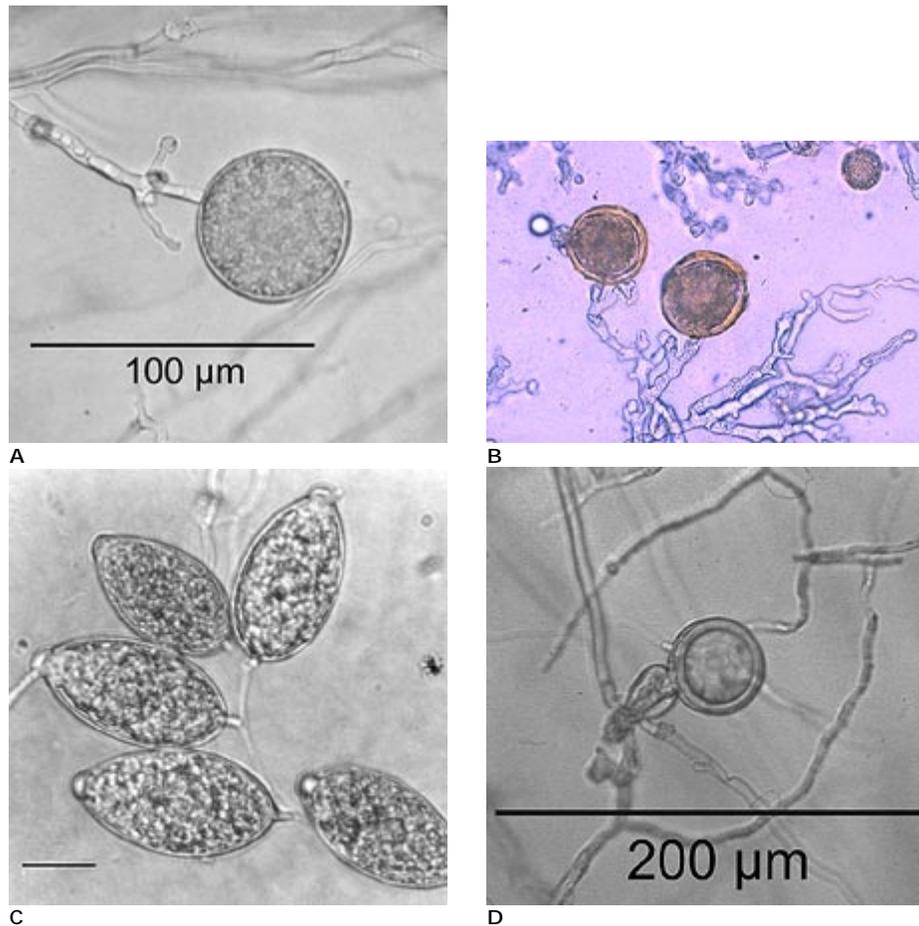


Fig. 34. Chlamydospores (A, B), sporangia (C, photo courtesy H. de Gruyter, Plant Protection Service, The Netherlands), oospore (D), and hyphae (on PARP) (B) of *P. ramorum*.

Molecularly, *P. ramorum* is unique at the ITS region and can be distinguished from all other known *Phytophthora* species (2,19,24). Based on ITS, the closest relative to *P. ramorum* is *P. lateralis*.

Pathogen Storage

P. ramorum has been successfully stored for extended periods of time in Germany, where the pathogen was discovered in 1993. The best method for storage is freezing in liquid nitrogen (S. Werres, *unpublished data*). Storage has also been successful on oatmeal agar in 20-ml tubes under paraffin oil (S. Werres, *unpublished data*), on cleared V8 slants in 20-ml tubes stored in the dark at 12°C, or on agar disks under water in tubes at room temperature. Cultures are replaced annually. *P. ramorum* can be stored on V8 slants at 15°C for several months, however, viability and pathogenicity decline.

Pathogenicity Tests

P. ramorum readily lends itself to a variety of pathogenicity tests. We have performed wound inoculations on trunks, stems, and leaves (18,19,24). Wound inoculations on trunks were completed by coring a hole into the bark, adding mycelium from a V8 plate, replacing the core, and covering the wound for 14 days. Wound inoculations on seedlings consisted of cutting a slit in the stem, adding mycelium from V8 and then wrapping the wound with parafilm. Wound inoculations onto leaves or green stems consisted of pinning a disk of mycelium to the plant part, spritzing with water, and then covering the plant with plastic to maintain high moisture levels for 3 to 7 days.

In addition, non-wounding inoculations can be done by pipetting or spraying a zoospore solution onto plant parts or by dipping plant tissue into a zoospore solution. Concentrations of 5,000 to 10,000 zoospores/ml have resulted in successful infection of plants using the dipping method (E. M. Hansen, *unpublished data*; D. Hüberli, *unpublished data*; J. Parke, *unpublished data*; P.

Tooley, *unpublished data*). Tissue should be kept at high humidity for 5 to 7 days after inoculation. *P. ramorum* mycelial disks on V8 agar readily produce sporangia in soil water (or to a lesser extent in distilled water) in 24 hours at 15 to 20°C. Soil water level should be even with, but not over the top of mycelial disks. Zoospore release is induced by placing a sporangial culture at 5°C for 30 minutes, and then returning to room temperature for 30 minutes.

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