Susceptibility of Oregon Forest Trees and Shrubs to *Phytophthora ramorum*: A Comparison of Artificial Inoculation and Natural Infection

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ABSTRACT

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Phytophthora ramorum is an invasive pathogen in some mixed-hardwood forests in California and southwestern Oregon, where it causes sudden oak death (SOD) on some members of *Fagaceae,* ramorum shoot dieback on some members of Ericaceae and conifers, and ramorum leaf blight on diverse hosts. We compared symptoms of *P. ramorum* infection resulting from four different artificial inoculation techniques with the symptoms of natural infection on 49 western forest trees and shrubs; 80% proved susceptible to one degree or another. No single inoculation method predicted the full range of symptoms observed in the field, but whole plant dip came closest. Detached-leaf-dip inoculation provided a rapid assay and permitted a reasonable assessment of susceptibility to leaf blight. Both leaf age and inoculum dose affected detached-leaf assays. SOD and dieback hosts often developed limited leaf symptoms, although the pattern of midrib and petiole necrosis was distinctive. Stem-wound inoculation of seedlings correlated with field symptoms for several hosts. The results suggested that additional conifer species may be damaged in the field. Log inoculation provided a realistic test of susceptibility to SOD, but was cumbersome and subject to seasonal variability. Pacific rhododendron, salmonberry, cascara, and poison oak were confirmed as hosts by completing Koch's postulates. Douglas-fir was most susceptible to shoot dieback shortly after budburst, with infection occurring at the bud.

Phytophthora ramorum is a recently established invasive pathogen in some mixed-hardwood forests of central coastal California (10,11) and extreme southwestern Oregon (5). It also is known from horticultural nurseries and landscape plantings in Europe (12) and recently was reported from a few nurseries in California and Oregon (6,9). Symptoms are dramatically different on different host species. The pathogen causes sudden oak death (SOD), characterized by lethal bole cankers, on some members of Fagaceae (oaks). On some members of Ericaceae and conifers, the disease is called ramorum shoot dieback, and a diverse group of hosts is susceptible to ramorum leaf blight (4). The North American forest host list includes at least 23 species in 12 families.

P. ramorum is a pathogen of aerial parts of woody plants. It produces deciduous sporangia on infected leaf and twig tissue of some hosts. It is recovered regularly from rain water in infected stands, and sporangia sprayed on leaves or boles of susceptible trees initiate new infections (J. Davidson, *unpublished*). It also is recovered from streams and soil in areas of infestation (3; E. M. Hansen, *unpublished*). Root infection has not been observed under field conditions, but planting into artificially infested potting mix (C. Lewis and J. L. Parke, *unpublished*), use of infested water for overhead irrigation, and rain splash from soil (J. Davidson and E. M. Hansen, *unpublished*) can lead to new infections.

The combination of broad host range, diverse symptoms, and aerial dispersal creates a diagnostic and disease management challenge. The pathogen is subject to state, federal, and international quarantines, and there is great concern about its potential spread to the large areas of known hosts in western forests beyond the current distribution, as well as to forests around the world where related plants grow. Only naturally infected host plant species are subject to quarantine regulation in the United States. Artificial inoculations reported here and elsewhere indicate that many more species are susceptible, at least under test conditions. These should be considered potential hosts should the pathogen spread.

Our goal was to predict, through artificial inoculation, the symptoms and impact of *P. ramorum* on some western forest trees that have not yet been exposed to the pathogen in nature. In this article, we compared symptoms of *P. ramorum* infection resulting from four different artificial inoculation techniques with the symptoms of natural infection on some forest trees and shrubs. In the process, we completed Koch's postulates for the Oregon forest hosts. Finally, we inoculated a range of forest tree species not yet reported as hosts to determine their susceptibility to the pathogen.

MATERIALS AND METHODS

Field collections and pathogen identification. Plants growing in association with diseased tanoaks in infested areas of southwestern Oregon were examined for leaf and stem symptoms suggestive of *P. ramorum* infection. Symptomatic tissues were collected for later isolation and molecular diagnosis. Isolations were attempted using *Phytophthora* selective agar (CARP; natamycin [Delvocid, DSM Food Specialties] at 10 ppm, Na-ampicillin at 200 ppm, rifampicin at 10 ppm, and commercial corn meal agar [CMA]). Isolation attempts were made from symptomatic and adjacent healthy tissue. Resultant *Phytophthora* colonies were transferred to corn meal agar (βCMA; commercial CMA + β-sitosterol at 30 ppm) for identification. *P. ramorum* was identified by its distinctive mycelium and production of chlamydospores and deciduous sporangia on solid medium (12). Duplicate samples were used for DNA extraction and polymerase chain reaction (PCR) amplification of the internal transcribed spacer (ITS) region of rDNA. The diagnostic primers and methods developed for *P. lateralis* by Winton and Hansen (14) were used. They also amplify the very closely related *P. ramorum*. The complete ITS rDNA sequence was obtained utilizing ITS 4 and ITS 5 primers (13) for at least one isolate from each host, and compared with published sequences.

Artificial inoculation. *P. ramorum* isolates for inoculation (Table 1) originally were recovered from diseased plants collected in the SOD outbreak areas in southwestern Oregon and California and maintained on βCMA at 18°C. These, and all other Oregon forest isolates, are A2 mating type, North American genotype (6). Long-term storage was on agar plugs in vials of water at room temperature. *P. ramorum*

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was reisolated from inoculated plants onto CARP. Mycelial inoculations used colonized agar plugs removed from the growing margin of colonies on βCMA. Sporangia for zoospore inoculations were produced on one-third-strength clarified V8 juice agar (66 ml of clarified V8 juice and 15 g of agar/liter). Plates with 2-week-old colonies bearing abundant sporangia were flooded with double-distilled (dd) H_2O and chilled for 1 to 3 h, then lightly scraped before decanting. Zoospore release began immediately. Zoospores and sporangia from three to four isolates were mixed for leaf-dip and plant-dip inoculations. Zoospore concentrations ranged from 1×10^4 to 2×10^5 per milliliter, depending on the test, or were diluted for the comparison of inoculum dose effects (see below).

Thirty-eight tree and four woody shrub species native to Oregon and three nonnative trees (chestnut, red oak, and pin oak) were tested (Table 2). Five different inoculation methods were compared, although not all species were tested with each method. Most tests used 1-year-old nursery-grown plants, usually with stem calipers about 5 mm. In most trials, three to five leaves, plants, or logs (see below) of each species were inoculated as replications, and one was treated as the uninoculated control. Most inoculation trials (except as noted below) were incubated in a growth chamber set for a 12-h light cycle and temperature of 17 to 20°C, with supplemental mist to maintain chamber humidity above 70%. Reisolation onto CARP was attempted from symptomatic tissues as well as from controls.

Inoculation methods. Zoospore spray inoculations were attempted on both detached leaves and intact plants. A suspension of swimming zoospores was sprayed to runoff on leaf surfaces using an atomizer. Control plants were sprayed with water only. Infection was erratic with zoospore sprays. Zoospores encysted during spraying and, while the cysts were viable, subsequent germ tube growth was observed to be perpendicular to the leaf surface. This inoculation method was not continued, and results are not presented.

Detached leaves were immersed in zoospore suspension (leaf dip) for about 3 s, allowed to drain briefly, and laid flat on moist paper towels in plastic crisper boxes for incubation. Symptoms were recorded after 7 days.

The influences of zoospore concentration and leaf age on symptom development were measured in three leaf-dip tests. In the first test, 4-month-old mature leaves of tanoak and myrtlewood (bay laurel) were compared with 1-month-old leaves that were fully expanded but still "softer" than the older leaves, at zoospore concentrations from 0 to 8.0×10^4 spores/ml. Foliar necrosis was estimated visually after 7 days. Results of two trials were similar, and only the data from the first are presented (Table 3).

In a second test, mature leaves of tanoak, rhododendron, evergreen huckleberry, madrone, and myrtlewood were dipped in fivefold dilutions of zoospore suspensions (2.4×10^3, 1.2×10^4, or 6.0×10^4 zoospores ml^{-1}) or sterile water (control). Five leaves of each species were inoculated at each concentration. Seven days after inoculation, leaves were photographed and the percent necrotic area measured with image analysis software (Assess; American Phytopathological Society Press, St. Paul, MN). Finally, leaf age effects on symptom development were measured in evergreen huckleberry, for which leaf age is correlated with leaf position. One evergreen huckleberry sprig (24 to 30 cm long) from each of five plants was removed, and 24 leaves in each sprig were individually dipped in zoospore inoculum (6×10^4 zoospores ml^{-1}) and evaluated for disease symptoms, keeping track of leaf position. Percent necrotic area of each leaf was measured using image analysis software. Leaves were divided into three age categories: the youngest (leaves 1 to 6), intermediate age (leaves 7 to 16), and mature (leaves 17 to 24). Statistical analysis was performed with SAS software (version 8.2; SAS Publishing, Cary, NC) using the PROC MIXED model, repeated measures analysis, treating leaves as repeated measures on each plant and allowing variances and covariances between age categories to vary. The Tukey-Kramer test was used to compare differences among means for the three leaf age categories.

Terminal portions of intact potted plants also were inoculated by dipping in zoospore suspension (plant dip). Inoculated portions of plants were enclosed in a plastic bag and not further disturbed. Bags were removed after 7 days; in most cases, leaves remained moist throughout from the combined effects of the original dip and transpired water vapor. Symptoms were recorded weekly for 3 to 5 weeks. Controls for both leaf-dip and plant-dip inoculations were dipped in water.

Additional plant-dip inoculation tests were made on potted Douglas-fir trees in order to determine the timing and location of initial infection. In separate tests, 3- and 4-year-old Douglas-fir trees, 1 to 2 m tall, were inoculated at several growth stages by dipping shoot tips in zoospore suspension and bagging the inoculated portions. Winter dormant trees were brought into the greenhouse at weekly intervals so that, at inoculation, they ranged from still fully dormant to fully flushed, with new growth elongated and new buds set. Eight phenological stages were inoculated: 1 = dormant, terminal buds tight; 2 = buds swelling but new needles not yet visible; 3 = bud burst, with new growth just emerging from bud scales; 4 = new shoot <2.5 cm long, needles not fully elongated; 5 = new growth 2.5 to 15 cm, needles elongated but new buds not yet differentiated; and 6 = new growth >15 cm, new buds set. In addition, immature female cones were inoculated while still upright, before pollination, and when pendant, after pollination. Two or three water-only controls, and 5 or, usually, 10 zoospore inoculations were made on each growth stage. Several inoculations were made on different shoots of each plant. The test was repeated, using a total of 25 trees. Symptoms were recorded after 3 weeks (test 1) or 4 weeks (test 2). Results were similar in the two tests; therefore, data were combined.

Stem inoculations were made on intact potted plants. A 6- to 10-mm-long transverse cut was made through the stem bark to the cambium and a portion of colonized CMA, about 2 mm^3, was inserted. The wound was wrapped with moist cheesecloth and then with aluminum foil. Control inoculations used uncolonized agar. There were three trials with most species tested at least twice. Plants were incubated for 3 weeks. The outer bark was scraped away to reveal the margins of lesions, and total lesion extent was recorded as the distance between the upper and lower lesion boundaries less the length of the inoculation wound.

Log tests utilized freshly cut bole sections from forest-grown trees. Logs were 10 to 15 cm in diameter and 1.0 to 1.5 m long. Three logs of each species were in-

Table 1. *Phytophthora ramorum* isolates used in inoculation tests

Isolate	Host	Date isolated	Source	Location	Tests
0-7	Tanoak	June 2000	D. Rizzo	Muir Woods, CA	Log
1004.1	Evergreen huckleberry	August 2001	E. Hansen	Curry County, OR	Log
2018.1	Tanoak	June 2001	E. Hansen	Curry County, OR	Log, stem, leaf dip, plant dip
2027.1	Tanoak	July 2001	E. Hansen	Curry County, OR	Log, stem, leaf dip, plant dip
2109	Tanoak	December 2001	J. Parke	Curry County, OR	Log, stem, leaf dip, plant dip
4123	Rhododendron	April 2001	E. Hansen	Curry County, OR	Leaf dip, plant dip
4163	Tanoak	August 2001	E. Hansen	Curry County, OR	Leaf dip, plant dip
4173	Evergreen huckleberry	August 2001	E. Hansen	Curry County, OR	Leaf dip, plant dip

oculated in a test. Log ends were sealed with wax to retard drying. Holes (0.5 cm in diameter) through the bark to the cambium were made with a cork borer. A matching plug of agar, either colonized by *Phytophthora* spp. or uncolonized for controls, was inserted into each hole and the bark piece replaced. Inoculation points were covered with wet cheese cloth and then aluminum foil held in place with tape. Four to six inoculations and one control inoculation were made in each log. Logs were incubated in large plastic bags for 5 weeks in the growth chamber or a greenhouse. After wrappings were removed, the outer bark was scraped away to reveal margins of any necrotic area. The lesion extent was measured horizontally and vertically. Lesion area was calculated as a diamond-shaped area less the inoculation wound: (horizontal measure – 0.5 cm) × (vertical measure – 0.5 cm)/2.

RESULTS

Hosts and symptoms in Oregon forests. *P. ramorum* was isolated and confirmed by diagnostic PCR (14) from symptomatic tanoak, evergreen huckleberry, myrtlewood, Pacific rhododendron, salmonberry, poison oak, and cascara plants from several locations near Brookings, OR. ITS DNA sequences were identical to published *P. ramorum* sequences (10,12). All infected plants were found growing in the immediate vicinity of a symptomatic tanoak tree. Symptoms on tanoak in Oregon were like those described in California (4,10), with bleeding, girdling bole cankers the most dramatic. Tanoak foliar symptoms were seen on understory trees, often associated with twig dieback, and on stump sprouts. Infected leaves usually had a black necrotic midrib and petiole.

Evergreen huckleberry was the most abundant host plant on the Oregon sites after tanoak, but infection symptoms were observed infrequently. Field symptoms included leaf blight and shoot dieback, as described in California (11). Myrtlewood is a common host in California (11), but only one infected sprout was confirmed in Oregon, despite the common presence of the tree on the infested sites in Oregon. The symptoms included limited leaf tip blight with a yellow margin, as described in California. One additional leaf from this same site was PCR positive, although isolation was unsuccessful.

Pacific rhododendron exhibited ramorum shoot dieback when growing beneath tanoaks with SOD in Oregon (5). It has not been reported to be infected in California. Infections appeared to originate in buds or flower clusters as well as leaves and resulted in progressive shoot dieback. New infections were observed following rain events in late spring. Leaf blight was seen mostly at the leaf tips and bases, with necrosis progressing along the midrib and down the petiole into stems, as well as moving from infected stem tissue into leaves through the petioles. Plants were occasionally killed, with stems 3 to 6 cm in diameter girdled.

Cascara was present but not abundant on most of the infested sites in Oregon, but *P. ramorum* was isolated only once from this host. Symptoms included leaf blight and dieback of basal sprouts. Salmonberry was infected at only one location, but it was not examined closely in the early investigations. Leaf blight was observed, characterized by marginal and interveinal leaf necrosis. Lesions initially were dark and

Table 2. Plant species inoculated

Family, genus, species	Common name
Angiosperms	
Aceraceae	
Acer circinatum Pursh	Vine maple
Acer macrophyllum Pursh	Bigleaf maple
Anacardiaceae	
Rhus diversiloba Torrey and A. Gray	Poison oak
Betulaceae	
Alnus rhombifolia Nutt.	White alder
Alnus rubra Bong.	Red alder
Corylus cornuta Marsh.	Hazelnut
Cornaceae	
Cornus nuttallii Aud.	Dogwood
Ericaceae	
Arbutus menziesii Pursh	Madrone
Arctostaphylos uva-ursi (L.) Spreng.	Kinnikinnick
Rhododendron macrophyllum G. Don	Pacific rhododendron
Vaccinium membranaceum Dougl.	Big huckleberry
Vaccinium ovatum Pursh	Evergreen huckleberry
Vaccinium parvifolium Smith	Red huckleberry
Fagaceae	
Castanea dentata (Marsh.) Borkh.	American chestnut
Castanopsis chrysophylla (Dougl.) DC.	Chinquapin
Lithocarpus densiflorus (Hook. & Arn)Rehder	Tanoak
Quercus chrysolepis Liebm.	Canyon live oak
Quercus garryana Dougl.	Oregon white oak
Quercus kelloggii Newberry	California black oak
Quercus rubra L.	Red oak
Quercus palustris Muench.	Pin oak
Lauraceae	
Umbellularia californica (Hook.&Arn.) Nutt.	Myrtlewood or California bay
Oleaceae	
Fraxinus latifolia Benth.	Oregon ash
Rhamnaceae	
Rhamnus purshiana DC.	Cascara
Rosaceae	
Prunus emarginata (Dougl.) Walp.	Bitter cherry
Rubus spectabilis Pursh	Salmonberry
Salicaceae	
Populus tremuloides Michx.	Quaking aspen
Populus trichocarpa T. & G.	Cottonwood
Populus trichocarpa × *P. deltoides*	Hybrid poplar
Salix hookeriana Barratt	Hooker's willow
Salix lasiandra Benth.	Pacific willow
Conifers	
Cupressaceae	
Chamaecyparis lawsoniana (A. Murray) Parl.	Port-Orford-cedar
Libocedrus decurrens Torr.	Incense cedar
Thuja plicata Donn.	Western red cedar
Pinaceae	
Abies concolor (Gord. & Glend.) Lindl.	White fir
Abies grandis (Dougl.) Forbes	Grand fir
Abies magnifica Murray	Red fir
Abies procera Rehder	Noble fir
Larix occidentalis Nutt.	Western larch
Picea sitchensis (Bong.) Carr.	Sitka spruce
Pinus contorta var. *contorta* Dougl.	Lodgepole pine
Pinus lambertiana Dougl.	Sugar pine
Pinus monticola Dougl.	Western white pine
Pinus ponderosa Dougl.	Ponderosa pine
Pseudotsuga menziesii (Mirbel) Franco	Douglas-fir
Tsuga heterophylla (Raf.) Sarg.	Western hemlock
Taxaceae	
Taxus brevifolia Nutt.	Pacific yew
Taxodiaceae	
Sequoia sempervirens (D. Don) Endl.	Coast redwood
Sequoia gigantea (Lindl.) Decne.	Sierra redwood

Table 3. Effect of *Phytophthora ramorum* zoospore concentration and leaf maturity on symptom development after leaf-dip inoculation

	Tanoak[y]		Myrtlewood[z]	
Zoospores/ml	**Mature**	**Young**	**Mature**	**Young**
8.6×10^4	10	70	5	100
8.6×10^3	5	75	0	90
8.6×10^2	10	20	0	0
8.6×10^1	0	0	0	0
0 (control)	0	0	0	0

[y] Mean percent midrib length necrotic ($n = 4$).
[z] Mean percent leaf area necrotic ($n = 4$)

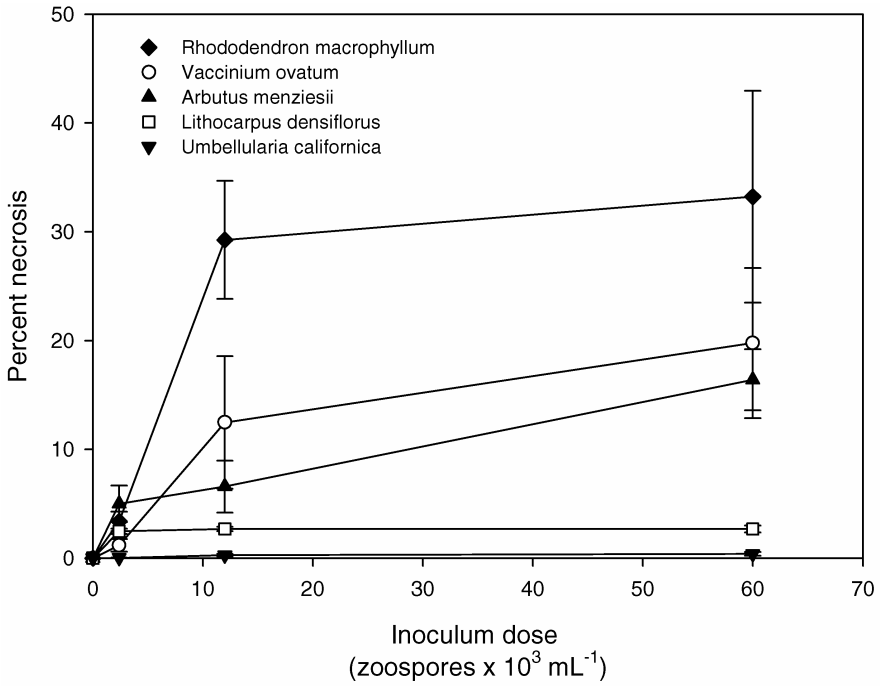

Fig. 1. Relationship between inoculum dose and response of five plant species to leaf-dip inoculation in suspensions of *Phytophthora ramorum* zoospores (0, 2,400, 12,000, or 60,000 zoospores ml^{-1}). Data points are means of five leaves per plant species; bars indicate standard error of the means.

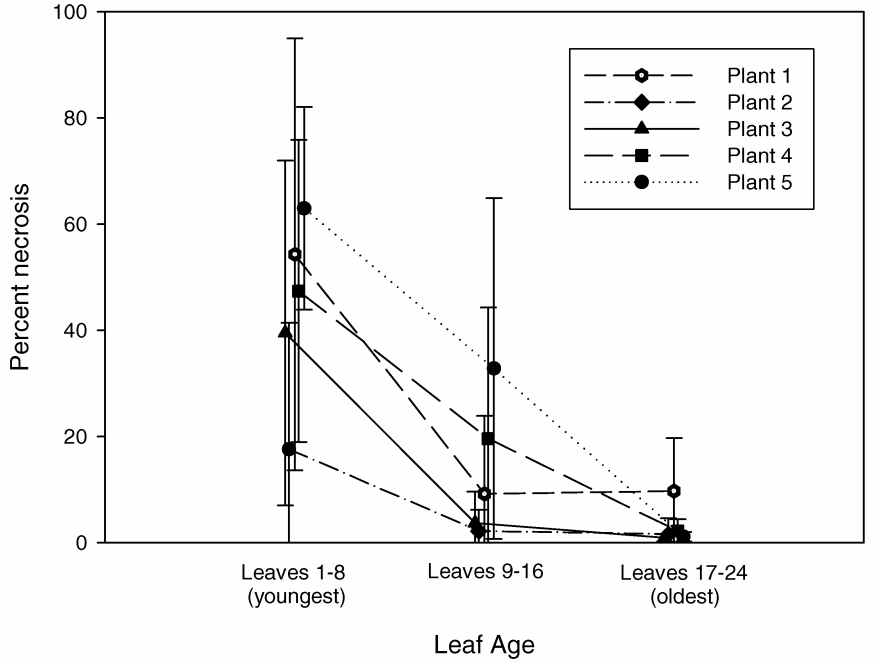

Fig. 2. Susceptibility of evergreen huckleberry leaves to *Phytophthora ramorum* in relation to leaf age (leaf position). Values shown are means ± standard deviation.

water-soaked and became brittle as they dried.

Infection of poison oak was observed twice, but the host is very infrequent on most infested sites in Oregon. The infected plants were twining around tanoak trees with bleeding cankers. The poison oak plants exhibited bleeding cankers up to about 2 cm long along their stems (up to 5 cm in diameter). *P. ramorum* was readily isolated from the cankers. No foliar symptoms were observed in the field.

Comparison of inoculation methods. In leaf-dip experiments with zoospores, both spore concentration and leaf age affected symptom development. In the first test (Table 3), symptom development consistently was greatest at the highest spore concentration and on immature leaves. The influence of leaf maturity was particularly striking with myrtlewood. Mature leaves developed the typical limited tip necrosis only at the highest spore concentration, but immature leaves were completely necrotic. In the second test (Fig. 1), Pacific rhododendron, evergreen huckleberry, and madrone developed foliar symptoms covering from 6 to 33% of leaf area at the higher inoculum doses (1.2 and 6.0×10^4 zoospores ml^{-1}). Tanoak developed only blackening of the petiole, and only a few tiny leaf spots were observed on myrtlewood, even at the highest inoculum dose. Analysis of variance (ANOVA; excluding the controls) showed a significant dose-response relationship for all species except myrtlewood. For the third test with evergreen huckleberry, mean percent necrosis for each leaf age category and plant is shown in Figure 2. Although percent necrosis was highly variable within each leaf age category and among individual plants, the youngest leaves were significantly more susceptible than either intermediate-aged leaves ($P < 0.008$) or mature leaves ($P < 0.01$). Intermediate-aged leaves were not significantly more susceptible than older leaves ($P < 0.34$).

Leaf-dip inoculation in zoospore suspension provided an effective, rapid first screen for susceptibility. Of 39 plant species tested, 31 developed symptoms of infection, including all known natural hosts that were tested (Table 4). *P. ramorum* was readily isolated from symptomatic leaves, but not from controls and not from nonsymptomatic portions of infected leaves. Symptoms often were visible at 3 days and did not increase beyond 7 days. Necrotic areas were usually limited to <30% of leaf area and did not expand. Species with greater leaf necrosis included California black oak and several species in the family Ericaceae. Symptoms from leaf-dip inoculation of ramorum leaf blight hosts generally matched symptoms observed in the field. In most cases, foliar necrosis developed from leaf-dip inoculation of ericaceous hosts susceptible to ramorum shoot dieback, and symptoms on

oak relatives susceptible to SOD were concentrated along the midrib and petiole.

Plant-dip inoculations were done on 39 species (Table 4). Foliar symptoms developed on 21 species, and infection progressed into stems and caused dieback or plant death on 10 species, including all of the known ramorum dieback and SOD hosts that were tested. Frequency of infection and extent of subsequent leaf necrosis generally was less than with leaf-dip inoculation, except on hosts that exhibited dieback. On susceptible members of Fagaceae and Ericaceae, infection progressed down the midrib and petiole into the stem, as observed in natural infections.

Stem-wound inoculations resulted in necrotic phloem lesions extending beyond the margins of the inoculation wound in 32 of 41 species (Table 4). Wound reactions from control inoculations never extended more than 1 mm beyond the inoculation point. In 19 species, at least one of the inoculated stems was girdled. *P. ramorum* was readily isolated from advancing lesion margins in nearly all cases. There was no significant difference (P = 0.16) in mean

Table 4. Artificial inoculation of 49 tree and shrub species with *Phytophthora ramorum*, using four different inoculation tests[u]

	Test			
Plant family, species[v]	**Leaf dip (%)[w]**	**Plant dip (%)[x]**	**Log[y]**	**Stem[z]**
Fagaceae				
American chestnut	…	…	…	2.8
Golden chinquapin	…	…	45.2 (13.1–77.4), *n* = 2	…
Tanoak ***	5 (0–10), *n* = 5	Dieback 54 (0–100), *n* = 6	109.2 (16.8–232.4), *n* = 5	6.8 de
Canyon live oak **	10 (0–100), *n* = 4	Dieback 22 (0–67), *n* = 3	11.0 (8.9–13.1), *n* = 2	2.0 ab
Oregon white oak	15 (0–50), *n* = 4	0, *n* = 5	11.4 (1.4–38.8), *n* = 4	0.8 ab
California black oak **	49 (14–100), *n* = 2	Dieback 33, *n* = 2	7.2 (5.7–9.9), *n* = 3	3.0 bc
Pin oak	…	…	1.0, *n* = 1	…
Red oak	…	…	…	3.0
Ericaceae				
Madrone **	34 (0–100), *n* = 5	Dieback 67 (33–100), *n* = 2	0.6 (0.0–1.3), *n* = 2	6.5 de
Pacific rhododendron *	27 (5–66), *n* = 4	Dieback 100, *n* = 1	…	…
Evergreen huckleberry ***	32 (0–100), *n* = 23	Dieback 100, *n* = 15	…	…
Kinnikinnick	8 (0–24), *n* = 2	…	…	…
Big huckleberry	100 (100), *n* = 2	…	…	…
Red huckleberry	80 (55–100), *n* = 2	…	…	…
Betulaceae and Salicaceae				
White alder	20, *n* = 1	10 (0–20), *n* = 2	…	0.0
Red alder	8 (0–35), *n* = 4	0, *n* = 2	9.8 (1.0–20.0), *n* = 4	0.9 ab
Hazelnut	20 (5–48), *n* = 3	5 (0–10), *n* = 2	…	0.2
Quaking aspen	2 (<1–5), *n* = 2	0, *n* = 1	…	0.0
Black cottonwood	2 (0–5), *n* = 3	0, *n* = 2	…	0.0 a
Hybrid poplar	…	…	…	0.0
Hooker's willow	<1, *n* = 1	0, *n* = 1	…	0.0
Pacific willow	0, *n* = 1	0, *n* = 1	…	0.0
Other families				
Vine maple	1 (0–61), *n* = 4	<1, *n* = 2	…	1.3
Bigleaf maple **	20 (0–70), *n* = 5	<1 (0–1), *n* = 2	2.2 (0.0–4.2), *n* = 4	2.0 ab
Pacific dogwood	100, *n* = 1	20, *n* = 2	…	0.9
Oregon ash	25, *n* = 1	10, *n* = 1	…	0.0
Bitter cherry	75, *n* = 1	1, *n* = 1	2.1 (0.6–0.4), *n* = 2	5.2 cd
Cascara *	20 (<1–60), *n* = 5	<1 (0–1), *n* = 2	0.3, *n* = 1	1.2 ab
Poison oak *	25 (10–50), *n* = 1	…	…	3.1
Salmonberry *	…	1 (0–25), *n* = 1	…	…
Oregon myrtlewood ***	1 (0–11), *n* = 8	<1 (0–1), *n* = 3	0.0, *n* = 1	1.0 ab
Pinaceae				
White fir	…	…	…	2.2
Grand fir	10, *n* = 1	Dieback, *n* = 2	…	1.1 ab
Red fir	…	0, *n* = 1	…	3.2
Noble fir	60, *n* = 1	0, *n* = 1	…	2.2 ab
Western larch	<1, *n* = 1	0, *n* = 1	…	6.5 de
Sitka spruce	<1–100, *n* = 2	0, *n* = 1	15.1 (2.7–36.8), *n* = 3	1.2 ab
Lodgepole pine	0, *n* = 1	0, *n* = 1	3.3 (0–6.7), *n* = 2	1.4 ab
Sugar pine	<1, *n* = 1	0, *n* = 1	…	0.7 ab
Western white pine	5, *n* = 1	0, *n* = 1	…	6.8 de
Ponderosa pine	0, *n* = 1	0, *n* = 1	…	0.03
Douglas-fir **	1 (0–5), *n* = 3	Dieback, *n* = 3	12.7 (2.6–34.1), *n* = 4	8.0 e
Western hemlock	20 (0–40), *n* = 2	0, *n* = 1	4.9 (1.7–10.2), *n* = 3	6.5 de
Other gymnosperms				
Port-Orford-cedar	0, *n* = 3	0, *n* = 1	24.5 (0.0–73.3), *n* = 3	0.2 a
Incense cedar	…	0, *n* = 1	…	0.0
Giant sequoia	…	…	…	0.0
Coast redwood **	30 (0–70), *n* = 2	Dieback, *n* = 1	0.1 (0.1–0.2), *n* = 2	0.2 a
Pacific yew	1 (0–5), *n* = 2	Dieback, *n* = 2	…	2.6
Western red cedar	0, *n* = 2	0, *n* = 1	0.1, *n* = 2	0.4 ab

[u] Three or more leaves, plants, or logs of each species were included in each test; *n* = number of times the test was performed.
[v] Naturally infected in Oregon (*), California (**), or in both states (***).
[w] Mean (range) necrotic leaf area.
[x] Mean (range) necrotic leaf area or, if dieback, percent plants.
[y] Mean (range) lesion area (cm^2).
[z] Mean lesion length (cm). Lesion lengths followed by different letters are significantly different (P = 0.05). Lengths not followed by a letter were not compared statistically.

lesion lengths between tests for the 24 species tested twice; therefore, data were combined for analysis (Table 5). Species tested only once, in the third test, were not included in the analysis but are presented in Table 4. There were significant differences in lesion length between species. Lesions on all of the tested natural SOD and ramorum dieback hosts (tanoak, California black oak, Douglas-fir, madrone, and redwood) were larger than control lesions, and lesions on all except black oak girdled the stem, although the species differed greatly in total lesion length. Several species in the Pinaceae family (hemlock, white pine, Douglas-fir, and larch) were as susceptible to stem-wound inoculation as tanoak, whereas species in the families Betulaceae and Salicaceae generally were resistant.

Log inoculations were made on 19 tree species; average phloem lesions larger than control inoculations formed in 15 species (Table 4). The log test was repeated five times, but with different combinations of species tested each time. Tests 3 and 4 were subjected to ANOVA, with very similar results; test 3 is representative (Table 6). There were no consistent differences in lesion area among the isolates of *P. ramorum* tested (four isolates in test 3, two in test 4), and all isolates produced significantly larger lesions than control inoculations ($P = 0.001$; *data not shown*).

There also were significant differences between some tree species in all tests, but the rank order varied between tests. With some species, there were large differences in lesion area between tests conducted at different times of year (Table 7). Tanoak, for example, formed larger lesions in November than at other test times. Results for five species that were included in at least four of the five log tests are presented in Table 7. Lesions on tanoak were the largest among the species tested in four of the tests.

Inoculation tests with known hosts. Artificial inoculations confirmed the susceptibility of all seven plant species from which *P. ramorum* has been isolated in Oregon. Inoculation of tanoak consistently induced large cankers in log inoculations (mean, 109.4 cm^2 after 5 weeks) and long, girdling bark lesions (mean lesion length, 6.6 cm) in the stem test. Symptoms on detached leaves were infrequent, and usually limited to necrotic midribs and petioles. In plant-dip inoculation, infection progressed into the stems and killed the seedlings.

Rhododendron and evergreen huckleberry symptoms from artificial inoculation, including leaf blight, stem dieback, and plant death, mimicked those seen in the field. *P. ramorum* was readily reisolated from symptomatic tissues.

Leaf- and plant-dip inoculation of myrtlewood seedlings induced very limited leaf spotting. Stem inoculations produced limited necrotic lesions on some plants (mean, 0.6 cm). No necrotic lesions developed from log inoculations. Myrtlewood appears to be more resistant to infection than most plant species.

Cascara plant-dip inoculation resulted in leaf blight symptoms similar to those in the field, but no shoot dieback. Stem inoculations of small plants resulted in limited necrotic lesions with a few plants girdled (mean lesion length, 1.0 cm); no cankers formed in log inoculations. *P. ramorum* was reisolated from inoculated necrotic leaves and inoculated stems, but not from healthy tissues.

Plant-dip inoculation of salmonberry resulted in dark, marginal necrotic blotches up to about 1 cm in diameter on leaves. They did not expand after 3 days, and infected tissue fragmented as it dried on the plant. *P. ramorum* was readily reisolated from lesions on inoculated plants, but not from asymptomatic tissue on the same leaves, and not from control plants.

Poison oak inoculations were done on detached stems and leaves. Intact plants were not available for testing. Following stem inoculation, bleeding bark lesions up to 3.9 cm long developed in 3 weeks. There was little or no circumferential growth. Infected phloem was reddish, and the margin of the infected area was clearly delimited. Xylem discoloration extended beyond the phloem lesion in both control and inoculated wounds. Leaf-dip inoculation resulted in interveinal leaf blight with a water-soaked appearance. Black necrotic areas also formed on the petioles. The pathogen was reisolated from necrotic lesions on inoculated stems and leaves, but not from uninoculated controls and not from nonsymptomatic leaf tissue on inoculated leaves.

Six species naturally infected in California but not found infected in Oregon also were included in the inoculation trials (Table 1). All of these trees grow on or close to SOD sites in Oregon. California black oak is infected and killed in California, where it grows in mixture with infected coast live oak (11). It was susceptible in all inoculation tests, with symptoms similar to but less severe than those on tanoak (log inoculation mean lesion area = 7.2 cm^2, stem lesion length = 2.7 cm). In the plant-dip test, foliar lesions formed primarily along the main veins and often extended down the midrib and petiole, then into the stem.

Canyon live oak was not a known host when our testing was done, but *P. ramorum* has since been isolated from several trees in California and susceptibility confirmed by inoculation (8). In our tests, it was particularly susceptible to plant-dip inoculation, with leaf necrosis progressing down petioles into stems and

Table 5. Analysis of variance for stem lesion lengths following wound inoculation of 24 tree species

Source	df	Ms	*F*	*P*
Main effects				
Test	1	778.8	2.03	0.16
Tree species	23	4,820.5	12.55	0.00
Residual	123	384.2	…	…
Total (corrected)	147	…	…	…

Table 6. Analysis of variance for bark lesion area following inoculation of logs of eight tree species with four isolates of *Phytophthora ramorum* in log test 3

Source	df	Ms	*F*	*P*
Main effects				
A: Tree species	7	9.10605E7	19.38	0.00
B: Isolate	3	1.19862E6	0.26	0.86
Residual	93	4.69953E6	…	…
Total (corrected)	103	…	…	…

Table 7. Mean bark lesion area for five tree species inoculated with *Phytophthora ramorum* in five tests at different times of year

	Mean bark lesion area (cm^2)				
Tree species	**Test 1, October 2001**	**Test 2, November 2001**	**Test 3, January 2002**	**Test 4, May 2002**	**Test 5, November 2002**
Bigleaf maple	0.0	1.8 (0.1–6.1)	…	4.2 (2.1–8.4)	2.7 (0.6–4.8)
Red alder	16.7 (2.0–28.5)	1.0 (0.7–1.8)	…	19.5 (6.3–38.2)	1.8 (1.0–4.5)
Tanoak	58.7 (17.0–245.7)	232.6 (171.1–343.6)	16.8 (6.2–31.2)	51.3 (14.6–126.0)	187.6 (62.6–331.3)
Oregon white oak	3.9 (0.1–21.5)	1.3 (0.0–6.4)	…	38.8 (0.2–94.1)	1.5 (0.5–3.1)
Douglas-fir	2.6 (0.2–14.6)	…	34.1 (16.4–61.4)	7.4 (4.2–11.1)	6.7 (3.0–10.1)

killing the plants. Distinctive lesions formed in log inoculations (11.0 cm^2). Lesions from stem inoculation (2.7 cm) were smaller than on tanoak.

Madrone is considered very susceptible in California, where it grows with other infected hosts. Leaf necrosis progresses into stem lesions that cause a shoot dieback (4). Similar symptoms developed in plant-dip inoculations. Madrone was very susceptible to stem inoculation (7.6-cm lesion length), but log inoculation resulted in strictly limited cankers (0.2 cm^2).

Bigleaf maple is reported to suffer ramorum leaf blight in California (11), although it has been difficult to isolate the pathogen from naturally infected symptomatic leaves. In leaf-dip and plant-dip inoculation, necrotic lesions formed on leaves, similar to but darker than those pictured in Davidson et al. (4) from the field. There was no progressive stem necrosis from plant-dip inoculation. Stem inoculation and log inoculation resulted in very limited bark lesions (1.5 and 1.1 cm^2, respectively).

Basal sprouts of coast redwood are infected in California, resulting in needle blight and twig dieback (7). Similar symptoms resulted from plant-dip inoculation, but redwood was resistant to log inoculation, and stem inoculation resulted in strictly limited lesions.

Douglas-fir has been reported as a host of *P. ramorum* in California (2), with twig dieback the primary symptom. Similar twig dieback was observed with plant-dip inoculation of seedlings that recently had burst bud. Scattered necrotic needles occasionally were observed on inoculated shoots at all growth stages, but shoot dieback only resulted from infections near the bud or at the base of young cones. *P. ramorum* regularly was isolated from dieback margins on inoculated shoots, but not from symptomless shoots. Dieback occurred in one stage-5 control shoot, and *P. ramorum* was isolated from the lesion. Dieback occurred on 20% of shoots inoculated before bud burst (stages 1 and 2), with buds killed and lesions extending 4 to 10 cm down the branch. After 3 weeks, the lesions were dark and sunken, and needles had fallen. Infection rates increased as new growth emerged (stage 3, 25%; stage 4, 35%; and stage 5, 75%), but there was no infection of fully expanded stage 6 shoots where a new terminal bud was set. In all cases where it could be determined, infection occurred at the base of the bud or into the bud scar after new growth was initiated. In stages 3 to 5, lesions usually developed both proximally and distally from the bud scars. In stage 6 inoculations, only new tissues were dipped in zoospores, and no infections resulted. Scales on young cones were susceptible (5 of 5 upright cones infected and 9 of 12 pendant cones infected), with lesions often growing down the cone stalk and into the supporting stem.

Douglas-fir also was very susceptible to stem-wound inoculation; lesions averaged 6.5 cm long after 3 weeks, and had girdled the stems. Log inoculation gave variable results, with lesions strictly limited in most tests but twice as large as lesions on tanoak in a January test (Table 7).

Inoculation of potential hosts. Thirty-six additional trees and shrubs, not known to be hosts for *P. ramorum*, were inoculated to assess potential susceptibility and symptoms. Symptoms were induced using one or more tests on 34 of the 36 species.

Oregon white oak, in the family Fagaceae, developed leaf blight symptoms from leaf-dip inoculation but not from plant-dip inoculation. Distinct cankers (11.3 cm^2) formed in log inoculations but stem inoculation resulted in strictly limited lesions that did not girdle the stems of the small plants. A few Oregon white oak trees grow within a few miles of the present Oregon *P. ramorum* infestation, but the main range of the tree is further north and inland. If *P. ramorum* naturally infects this tree, we expect that symptoms will be limited to leaf blight.

Chinquapin was tested by log inoculation only, because seedlings were not available. Large, expanding cankers formed on the logs, similar to cankers on inoculated tanoak. Chinquapin trees are not present at the infested sites in Oregon, but are found in the vicinity.

Three species of Betulaceae were tested, and two exhibited leaf blight. Detached leaves of red alder developed limited leaf blight symptoms, but no symptoms resulted from the more realistic plant-dip test. Small cankers did form in log inoculations. Red alder is present on several infested sites in Oregon but has not been found infected. It appears to be resistant under field conditions. White alder, in contrast, developed limited leaf blight symptoms in plant-dip inoculations, and may be susceptible to leaf blight in the field. Hazelnut also developed symptoms of leaf blight in detached-leaf and intact-plant inoculations, although lesion size was very limited. It may be susceptible under field conditions. It subsequently has been reported to be infected in California (P. Maloney and D. Rizzo, *personal communication*). Species of willow and poplar (Salicaceae) were the most resistant to artificial inoculation of any species tested and probably will be resistant in the field.

Vine maple, Pacific dogwood, Oregon ash, and bittercherry all developed limited leaf blight symptoms in plant-dip inoculations, and may be susceptible to ramorum leaf blight under field conditions. Bittercherry also was very susceptible to stem inoculation.

Fifteen gymnosperm species were tested, and only four (Douglas-fir, grand fir, coast redwood, and Pacific yew) developed symptoms in plant-dip inoculation. The family Pinaceae, in particular, seem to have a limited period of susceptibility to dieback, shortly after bud burst in the spring, and inoculation of mature tissues may have contributed to the apparent resistant reactions of several *Abies* spp. Scattered needle infections were observed, but only grand fir was consistently susceptible to twig dieback in repeated inoculations. The pines tested developed no symptoms in plant-dip inoculations, although western white pine was very susceptible to stem inoculation, as was western hemlock. Four conifers were tested by log inoculations, and all were susceptible to one degree or another. Port-Orford-cedar gave inconsistent log reactions (similar to Douglas-fir).

DISCUSSION

We tested four different inoculation methods, comparing symptoms of *P. ramorum* infection on seedlings and logs of several native trees and shrubs with symptoms observed from natural infection in the forests of Oregon and California. Plant-dip inoculation, in which apical portions of intact seedlings were immersed briefly in zoospore inoculum and then incubated several weeks for symptom development, best mimicked the field susceptibility of the same species. This method allows uniform exposure of plant parts to the same infective propagules presumed active in nature. Artificial wounding is not required, and zoospores run off or concentrate according to the surface chemistry and topography of the plant surfaces. Plant-dip inoculation allows expression of both limited foliar symptoms and progressive shoot blight. Disadvantages include the rather cumbersome handling of individual plants during inoculation, and challenges of quantification of effective inoculum as well as symptom expression.

Fewer species develop ramorum leaf blight with plant-dip inoculation than with leaf-dip inoculation. We presume that this is the result of lower effective inoculum levels on leaf surfaces with plant-dip inoculation. The results more closely approximate susceptibility in the field than the predictions from leaf-dip inoculation. All of the tested natural hosts were susceptible to plant-dip inoculation. Tanoak developed limited leaf necrosis that progressed into twigs and stems, killing the seedlings. In the field, foliar infection is similarly limited, and shoot dieback is observed, although it is not generally progressive. Foliar and dieback symptoms similar to those observed in the field developed on inoculated madrone, huckleberry, and Pacific rhododendron seedlings. Plant-dip inoculation did not indicate a species' susceptibility to bole cankers (SOD), except that all SOD hosts tested also developed progressive dieback with plant-dip inoculation. Madrone, however, also developed dieback symptoms with both inoculation and natural infection, but was not susceptible to direct bole inoculation, and

bole cankers have not been observed in the field.

Leaf-dip inoculation is a quick, reliable, and quantifiable indicator of potential susceptibility to ramorum leaf blight. Many species developed substantial leaf necrosis in leaf-dip inoculation but few or no leaf symptoms from plant-dip inoculation when exposed to the same nominal spore load. The difference presumably results from a higher effective inoculum dose as well as more favorable incubation conditions available to individually treated leaves compared with dipped intact seedlings. Unlike intact plants, detached leaves also provide a wound for pathogen entry. Leaf age also can affect disease susceptibility; therefore, care must be taken to select leaves that best represent those of interest in the field. Leaf age effects on susceptibility to *P. ramorum* could contribute to variation in host susceptibility reported by different researchers. With conifers, excised shoots were dipped, not individual needles. Needle symptoms were variable and difficult to interpret. For several important hosts, foliar susceptibility does not reflect the impact of *P. ramorum* on individual trees or on the disease epidemic in the forest. Tanoak leaves are susceptible, but lesions usually are limited to petiole and midrib, despite the extreme susceptibility of the tree to bole cankers. Myrtlewood is even more resistant to leaf infection, but the limited tip lesions that do form at high inoculum levels produce abundant sporangia over extended times, making this tree a key source of inoculum in the forest epidemic in California.

Results of seedling stem wound inoculations of known hosts generally were congruent with field observations, with lesions not significantly different from the controls on foliar hosts, and significantly larger lesions on dieback and SOD hosts. Douglas-fir was most susceptible, followed by several other conifers. Of these, western white pine and western larch do not grow near infested forests, but western hemlock does, although it is not common in the immediate vicinity of any known infections. Coast redwood was resistant to stem-wound (and log) inoculation, despite reports of its susceptibility in California (7).

Log inoculations of broadleaf trees seemed to predict forest susceptibility to SOD. Only members of the family *Fagaceae* developed lesions that averaged >100 cm^2 in area, and Oregon white oak, present but not observed to be infected in outbreak areas, produced much smaller lesions than tanoak. Other species susceptible only to leaf blight or dieback in the field developed much smaller lesions or were not susceptible at all to bark-wound inoculation. The reactions of some of the conifers tested were extremely variable, however, and suggested potential susceptibility to SOD at least at some times of the year. Lesions on tanoak logs harvested in January were smaller than on tanoak logs harvested in March, September, or November, whereas lesions on Douglas-fir logs inoculated in January were larger than at other times of the year. In the January trial, lesion area on Douglas-fir was about twice that on tanoak. In the forest, however, only limited shoot dieback (in California) or no symptoms (in Oregon) have been observed. Brasier and Kirk (1) observed similar seasonal variation in susceptibility of various trees to log inoculations with other *Phytophthora* spp. They concluded that trees in the United Kingdom were most susceptible in late summer and fall, and essentially immune in April.

In conclusion, plant-dip inoculation provides the best prediction of susceptibility to the range of diseases caused by *P. ramorum* on forest plants. Dipping the shoots of intact plants in zoospore suspension detects both foliar and dieback infections while avoiding artificial wounding. It cannot predict susceptibility to bole cankers, except that all known SOD hosts develop progressive dieback following plant-dip inoculation.

These inoculation and reisolation results confirm the susceptibility of Pacific rhododendron, poison oak, salmonberry, and cascara. Field infection of these species was reported previously (4).

Interpretation of artificial inoculation results with tree species is often difficult. Practicality often forces inoculation of seedlings; however, if the disease in question affects mature trees, then potential juvenile susceptibility or resistance must be resolved. The small size and, frequently, the succulence of juvenile tissues may differ importantly from infection courts on mature trees. For some purposes, such as testing the efficacy of fungicides, these differences may not matter. However, when trying to predict the impacts of an invasive pathogen on forests not yet exposed, great caution is required. The problems are compounded with *P. ramorum*, because of its ability to cause very different disease symptoms on many plant species. The present inoculation results, and others yet to be published, indicate that about three-quarters of all woody plant species tested are susceptible to *P. ramorum* to one degree or another, but this likely overstates the threat to forest vegetation. The proportion subject to bole cankers or dieback is much smaller.

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