



Phytophthora leaf blight – a new disease of California wax-myrtle (*Morella californica*) in Oregon, USA caused by a *Phytophthora* species †Melodie L. Putnam^{1*}, Maryna Serdani¹, Marc Curtis¹, and Samson Angima²¹Oregon State University, 1089 Cordley Hall, Corvallis, OR, USA²Oregon State University Lincoln County Extension, 29 SE 2nd St., Newport, OR, USA

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Abstract

In spring, 2009, the Oregon State University Plant Clinic received reports of severe defoliation of California wax-myrtle plants (*Morella californica* (Cham. & Schlecht.) Wilbur) on the north-central coast of Oregon, in western North America. Isolations from necrotic leaf tissue yielded an organism which, from morphological characteristics and a genus-specific enzyme-linked immunosorbent assay, was identified as a species of the genus *Phytophthora*. Total DNA was extracted from hyphal tip-derived cultures from leaf or twig tissue and subjected to a polymerase chain reaction process aimed at species identification. Sequencing techniques revealed a $\geq 99.7\%$ match with *P. syringae* although our isolates differed from published descriptions of this species in some respects. Inoculation of healthy plants with cultured mycelium resulted in symptoms similar to those originally observed in the field, and reisolations produced colonies of the same organism. This is the first report of a species of *Phytophthora* causing disease in *M. californica*. Leaf blight of California wax-myrtle is now widespread on the north-central coast of Oregon. This disease is serious and is adversely affecting the health of this native understory species which is frequently used for amenity plantings.

Keywords: *Morella californica*; native plants; new host records; *Phytophthora syringae*; plant diseases.† Based on a paper presented at the fifth meeting of the IUFRO working party S07-02-09, *Phytophthora Diseases in Forests and Natural Ecosystems*, 7 – 12 March 2010, Auckland and Rotorua, New Zealand.**Introduction**

Morella californica (Cham. & Schlecht.) Wilbur (syn. *Myrica californica*), commonly known as California wax-myrtle, is an understory plant found in the western coastal region of the United States of America (USA). Its geographical range in the USA extends from southern Washington State to southern California. One population has been found on Vancouver Island, British Columbia, Canada (Figure 1).

Morella californica is a large evergreen shrub or small tree, often multi-stemmed, with a maximum size of around 10 m. In undisturbed areas, it grows in mesic sites at elevations below 165 m. It is found under canopies formed by species of *Pinus*, *Abies* and broadleaf trees such as *Salix* and *Alnus*. Associated understory species are *Gaultheria shallon* Pursh, *Rhododendron macrophyllum* D. Don ex G. Don, *Rhododendron* × *columbianum* (Piper) Harmaja, and *Vaccinium ovatum* Pursh. The fruit, consisting of small purplish berries that ripen in the autumn, is consumed

by native wildlife, especially migrating birds (Tietz & Johnson, 2007). Because the evergreen leaves are tolerant of wind and salt spray, *Morella californica* is commonly used for ornamental planting and for screens and hedges in coastal regions.

Morella californica is afflicted with few foliar diseases. A rust fungus, *Cronartium comptoniae* Arthur, occurs infrequently in Oregon. The leaf infecting fungi *Gnomonia myrica* Cook & Ellis, *Lophodermium foliicola* (Fr.) P. F. Cannon & Minter, *L. hysterioides* (Pers.) Sacc, and *Phyllosticta myrica* Cooke are documented on leaves from California (Farr & Rossman).



FIGURE 1: Distribution of *Morella californica* in North America. Note the one population on Vancouver Island, Canada. Arrow indicates the area of Oregon, USA in which diseased plants were first discovered. Map courtesy of the US Geological Survey.

In March 2009, ornamental *Morella californica* plants in Lincoln City, on the north-central Oregon coast, were found to be severely defoliated. Samples collected during the following months were submitted to the Oregon State University Plant Clinic for diagnosis. This is a brief report on the results of the investigation into the etiology of this disease.

Materials and Methods

Sample Collections

Healthy Plants

Seven healthy *Morella californica* plants in 4-L pots were obtained from a commercial nursery in Newport, Oregon.

Affected Tissues

March 2009

Morella californica branches with symptomatic leaves were collected from a home garden in Lincoln City, north-central Oregon coast, in Lincoln County, USA.

May 2009

Branches from two affected *Morella californica* specimens were collected from the same Lincoln City site as in March 2009.

June 2009

Additional branches of symptomatic *Morella californica* plants were collected from five sites in Lincoln County: Lincoln City (two plants from the same site as the March and May collections); the town of Glededen Beach (two sites); and Salishan spit (two sites). Branches with symptomatic leaves were also collected from one site in neighbouring Benton County, an inland county in central western Oregon, USA.

July 2009

One sample of *Morella californica* with necrotic leaf lesions was collected from near the town of Florence, in Lane County, central Oregon coast, USA.

Samples of symptomatic leaves were also collected from neighbouring plants of other species growing near affected *Morella californica* plants. Of the two Glededen Beach sites, from one we collected branches of *Ceanothus* sp. with twig dieback and leaf necrosis; from the other site we sampled *Vaccinium ovatum* leaves with round gray lesions. In Lincoln City, foliage from *V. ovatum*, *Cedrus deodara* (Roxb. ex D. Don) G. Don, and *Salix* sp. showing brown necrotic lesions were also collected. All samples were stored at 4 °C until processed, generally a day or two later.

Isolations

Leaves of affected *Morella californica* collected from the Lincoln City site in March, 2009 were surface-disinfested for 3 minutes in 0.6% sodium hypochlorite (NaOCl), rinsed, blotted, and allowed to dry under a sterile airflow. Tissue excised from lesion margins was placed onto two non-selective fungal media: water agar and half-strength potato dextrose agar (½PDA) and a medium semi-selective for the oomycete genera *Phytophthora* and *Pythium*. A modified version of the PARP medium devised by Jeffers and Martin (1986) was used. It was prepared as follows: distilled water with cornmeal agar (17 g/L) and the fungicide dicloran (2,6-dichloro-4-nitroaniline; Aldrich Chemical) (10 mg/L) was autoclaved and allowed to cool to 50 °C before adding Delvolid® (Gist-Brocades), a product containing 50% pimaricin and 50% lactose (20 mg/L); rifampicin (10 mg/L) and ampicillin (200 mg/L). All plates were incubated at 20 °C and examined after three and seven days in darkness.

Additionally, surface-disinfested symptomatic leaf tissue was triturated in a few drops of sterile deionised water with a sterile mortar and pestle and streaked to King's medium B, which supports bacterial growth (Schaad et al., 2001). The bacterial plates were incubated at 20 °C in ambient room light and examined after two days.

Symptomatic leaf and twig tissues from the May, June and July collections were plated onto water agar and modified PARP. These cultures were incubated in the dark at 10 °C and examined after three and seven days.

Hyphal tips from all putative *Phytophthora* species were taken for subculturing and maintenance on clarified 10% V8 juice agar. (To 340 mL V8 juice was added 5 g CaCO₃ to neutralise the acidity; the mixture was stirred for 15 minutes, and then centrifuged to concentrate the solids. The supernatant was poured off and 100 mL was added to 900 mL deionised water and 15 g agar, then autoclaved.) Developing colonies were used for morphological comparisons. Sporangia production was induced by removing 5-mm diameter plugs from the edge of five-day-old colonies and placing in a sterile 10-cm Petri plate, four plugs per plate and two plates per isolate. Water was added up to the surface of the plugs. In separate experiments, filtered stream water, 1% soil extract water, or cold (4 °C) non-sterile deionised water were added to the plugs. Stream water was collected from Oak Creek, Corvallis, OR, and filtered through a 5-µm pore size filter prior to use; soil extract water was created by mixing 10 g of air dry clay-loam native soil with 990 mL tap water, decanting after three days, and passing through a coarse filter to remove particulates. The water was then passed through a 5-µm filter prior to use. The plug plates were incubated in the dark at 10 °C and examined daily for

the presence of sporangia. In an attempt to observe sexual structures, cultures were grown on clarified V8 agar to which 1 g/L wheat germ oil was added (Doster & Bostock, 1988). Plates were incubated in the dark at 10 °C and were examined at two, four, and eight weeks for the presence of oogonia and antheridia.

Serological assay

As a matter of routine, symptomatic leaf tissue from all samples was subjected to a double antibody sandwich enzyme-linked immunosorbent assay (ELISA) specific to the genus *Phytophthora* (Agdia Inc., Elkhart, IN, USA). Briefly, leaf tissue triturated in buffer was added to a *Phytophthora*-specific antibody which had been adsorbed to polystyrene microtitre wells. The antigenic determinants of *Phytophthora* spp., if present, would bind to the antiserum-coated wells. The wells were washed after incubation, and a second antibody chemically linked with an enzyme was added to the wells. After further incubation and washing, a substrate was added to the wells. A positive reaction of the substrate with the enzyme produces a yellow colour. All reactions were quantified using a spectrophotometer (Molecular Devices, USA).

DNA analysis

Total genomic DNA was extracted using a FastDNA® Spin kit (Q•BIOgene, USA) from 100 mg of hyphal tip-derived cultures originally obtained from leaves collected from Florence, one of the Salishan spit sites, and from the March date from the Lincoln City site; and from twig tissue collected in May from Lincoln City. For each sample, an aliquot of 2 µL was used for a polymerase chain reaction (PCR) using DC6 and ITS4 primers specific for Pythiales and Peronosporales (Cook et al., 2000). The PCR mixture contained a buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl and 2 mM MgCl₂); 200 µM each of the deoxyribonucleotides dATP, dCTP, dGTP, dTTP; 1 unit of Taq-polymerase (Invitrogen, USA); 0.4 mg/mL bovine serum albumin; and 0.33 µM of each primer in a final volume of 30 µL. Thermalcycler conditions were 94 °C for 2 minutes 30 seconds; followed by 38 cycles of 94 °C for 25 seconds; 55 °C for 40 seconds; and 72 °C for 1 minute 20 seconds. Sequencing was conducted at the Center for Genome Research and Biocomputing (Oregon State University, Corvallis, Oregon, USA). The PCR product (15 µL) was prepared for sequencing by incubating for 40 min at 37 °C with a buffer (10 mM Tris-HCl, pH 7.5, 10 mM MgCl₂); 0.1 mg/mL bovine serum albumin; 8 units of exonuclease; and 0.25 units of shrimp alkaline phosphatase (SAP); in a final volume of 20 µL. Exonuclease and SAP were inactivated by incubation at 80 °C for 20 minutes. The PCR product was sequenced using the DC6 and the ITS4 primers which provided an overlapping sequence within the ITS region. Consensus sequences were compared with sequences in the *Phytophthora*-ID database

(<http://phytophthora-id.org/>) using ITS-BLAST (Basic Local Alignment Search Tool).

Pathogenicity testing

To maximise the potential for infection, inoculations were performed on both wounded and intact leaves of healthy *Morella californica* plants grown in 4-L pots; each experiment used two isolates, one derived from leaf tissue and the other from twig tissue. Inoculum consisted of clarified V8 agar plugs of 10-day-old hyphal tip cultures. For the wounding experiment, agar plugs (7 mm in diameter) were taken from colony edges and one plug was secured to each of nine leaves of one plant using a new thumb tack pushed through the agar and into the lamina, wounding the leaf. Each leaf was considered a replicate. Sterile agar plugs (7 mm in diameter; one per leaf) were attached in a similar way to three leaves of each of two control plants. All plants were sprayed with water, enclosed in a plastic bag and incubated in the dark at 4 °C for four days, then moved to a room with natural light at 21 °C. All treated leaves were examined 11 days after inoculation, and leaf lesion width and length were measured. Tissue from each inoculated and control leaf was plated to fresh modified PARP in an attempt to recover the organism. Isolation plates were incubated in the dark at 10 °C and examined at three and seven days.

A second experiment was conducted as above on separate plants except without wounding (one plant per isolate). Agar plugs were secured to each of nine leaves for each isolate with a strip of Parafilm; one control plant received sterile agar plugs on each of nine leaves, and again tissue from each inoculated and control leaf was plated to modified PARP as above.

Inoculated leaves were examined for sexual structures

four and eight weeks post-inoculation. Tissue not used for reisolation was retained and stored in plastic at 4 °C until examined. Small (5 mm square) pieces of symptomatic tissue from nine different leaves were immersed in 3% KOH and gently warmed until clarified. The tissues were then removed, blotted, and stained with 1% methyl blue in lactoglycerol and examined with a light microscope at 200× and 400× for oogonia and antheridia.

Results and Discussion

Morella californica occupies a unique niche in low-elevation western United States coastal regions as a large shrub that provides cover and food for migrating birds (Tietz & Johnson, 2007), and is valued as and widely used for amenity planting. Diseased plants of *M. californica* were discovered in an area extending 120 km along the central coast of Oregon in the western United States. Symptoms were severe in natural settings and in ornamental plantings, destroying their aesthetic value.

Symptoms

Affected *Morella californica* leaves had brown to dark charcoal colored blotches with diffuse margins which eventually spread across the lamina and petiole before abscission occurred. Both cultured and wild plants showed these symptoms (Figures 2 and 3, respectively). Leaf necrosis and defoliation were severe on lower branches, becoming less so on upper branches. Occasionally the branch near an affected petiole exhibited a small dark canker. Branch dieback in the absence of a canker appeared to be the result of repeated defoliation. Plants in deep shade exhibited greater defoliation than those in full sun.



FIGURE 2: Cultivated *M. californica* plant showing necrosis of leaves on lower branches (A); and Cultivated plant showing severe defoliation (B).



FIGURE 3: *Morella californica* plant in a wild setting showing leaf necrosis and defoliation of lower branches (A); and dark, spreading lesion at the tip of an infected leaf (B).

Isolations

Symptoms of affected *Morella californica* leaves were dissimilar to any diseases reported for this host; accordingly tissues from the first collection date were plated for fungi, oomycetes, and bacteria. The common saprophytes *Penicillium*, *Cladosporium*, and *Alternaria* were recovered from tissue on water agar and PDA; there was no growth on the modified PARP or the bacteriological medium. The inability to recover *Phytophthora* spp. from the PARP was likely due to the incubation temperature (20 °C); the isolates eventually recovered did not grow well at that temperature and all further isolations were incubated at 10 °C.

Diseases caused by *Phytophthora* spp. are frequent in western Oregon, and as a matter of course symptomatic leaves were tested with a *Phytophthora*-specific serological assay. Since the results of this test were positive, isolations from May, June and July sample collections were aimed at isolation of *Phytophthora* spp.. A *Phytophthora* sp. was recovered from *M. californica* leaves (plated to modified PARP and incubated at 10 °C) at all sites except Benton County, an inland county. One isolate was recovered from necrotic twig tissue collected in May from Lincoln City.

Four isolates of an organism recovered from naturally infected *Morella californica* plants showed morphological characteristics (appearance on modified PARP, the presence of coenocytic hyphae, and the production of sporangia in which zoospores are released in the absence of a vesicle) consistent with those of the genus *Phytophthora* (Erwin & Ribeiro, 1996). Colonies growing on clarified V8 agar had a stellate appearance (Figure 4). The hyphae possessed abundant irregularly globose to ellipsoid catenulate hyphal swellings (Figure 5). Few sporangia

were formed with any of the water treatments; those produced in Oak Creek water were barely papillate, sympodial, ovoid or obpyriform, and averaged (33.6 – 48 µm) 40.1 µm x 33.5 (25.6 – 41.6 µm) with a short pedicel (~ 3 µm). Sporangia were caducous, and attachment was central or slightly off-centre (Figure 6). No chlamydospores or sexual structures were observed in culture or in infected host tissue.

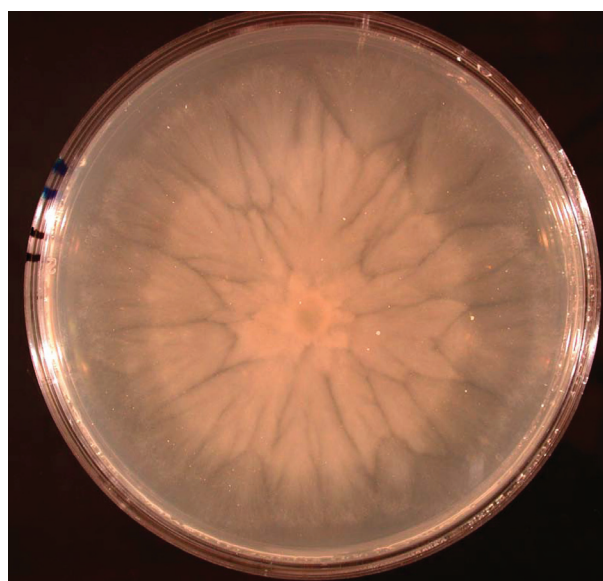


FIGURE 4: A 10-day-old culture of *Phytophthora* sp. isolated from an affected *Morella californica* leaf. The medium was clarified V8 juice agar.

Serological assay

Symptomatic tissue from all *Morella californica* leaves collected from all dates and all locations except the

Benton County site (collected in June, 2009) gave a positive reaction with the *Phytophthora*-specific ELISA. No *Phytophthora* sp. was detected in necrotic leaf tissue of collected plant species other than *M. californica* when assayed using ELISA.

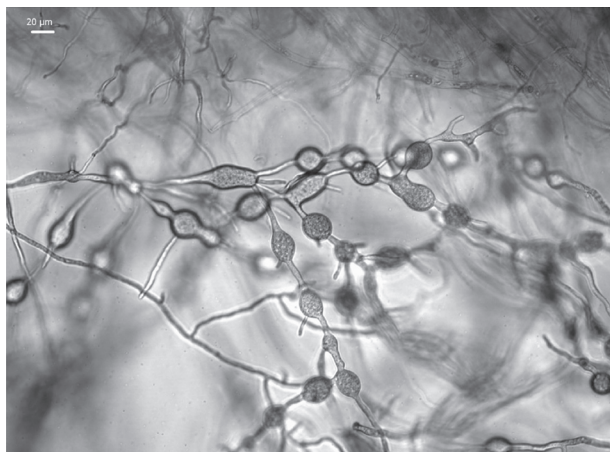


FIGURE 5: Hyphal swellings in solid agar of *Phytophthora* species isolated from *Morella californica*.

DNA sequencing

DNA extracts of hyphal tip-derived cultures, obtained from three symptomatic *Morella californica* leaves and one twig, representing three sites and two counties yielded a single PCR product of approximately 1400 bp. Consensus sequences for each of the isolates showed $\geq 99.7\%$ conformity with *Phytophthora syringae* (GenBank accession: AY787032.1) over 813 nucleotides, with one or two nucleotide mismatches.

Pathogenicity testing

Sporangial production was insufficient for inoculation via zoospores, hence inoculations were performed using mycelium in agar. We inoculated four individual healthy plants: wounded and unwounded leaves using a leaf isolate; and wounded and unwounded leaves using a stem isolate. Thirty-six separate leaves were inoculated in total. Fifteen separate leaves on three healthy plants received non-colonised agar plugs as controls. In the experiment using wounded leaves, dark spreading lesions similar to those observed on leaves of naturally affected plants were apparent on all inoculated leaves but not on any of the control leaves 11 days after inoculation. In the experiment using wounded leaves, lesions averaged 13 x 17 mm (leaf isolate) and 10 x 12 mm (stem isolate). Average lesion size on unwounded leaves was 4 x 7 mm (leaf isolate) and 14 x 18 mm (stem isolate), showing wounding was not necessary for infection. Four weeks post-inoculation, leaves which had been wounded were blackened, necrotic and starting to abscise. *Phytophthora* sp. was isolated from inoculated leaves but not from control leaves, which did not show similar lesions and did not abscise. Plants inoculated without wounding showed symptoms on all inoculated leaves 11 days post-inoculation (Figure 7). For both experiments and both isolates, *Phytophthora* was recovered from symptomatic leaves but not from control leaves; isolates were identified on the basis of their morphology. Inoculation, production of symptoms similar to those originally observed, and recovery of the inoculated organism from symptomatic tissue is considered proof of pathogenicity for an organism, hence we have shown that our two isolates of *Phytophthora* sp. are pathogenic to and cause disease on *M. californica*.



FIGURE 6: Sporangia (in water) of *Phytophthora* sp. isolated from *M. californica*. The bars each represent 20 μ m.

Identification

Although DNA analysis showed similarities with *Phytophthora syringae*, isolates collected from *Morella californica* differed morphologically from the description of the organism as given by Waterhouse & Waterston (1964) and may represent a new species. Mycelial growth on clarified V8 agar was stellate, rather than roseate. Sporangia were more difficult to produce than with *P. syringae*, and those of an isolate from *M. californica* were shorter in length (average of 40.1 µm) than those described for *P. syringae* (57 µm average). *Phytophthora syringae* is described as producing sexual structures abundantly in culture and in host tissue, but none were observed with the isolates from *M. californica* in either cultures or inoculated leaves after eight weeks. Finally, *P. syringae* has non-caducous sporangia, whereas those from *M. californica* were caducous. Further morphological and molecular characterisation of the *M. californica* isolates is in progress and it is hoped that this will clarify their relationship with *P. syringae*.



FIGURE 7: Necrosis on a leaf inoculated with a colonised agar plug secured with Parafilm.

Conclusions

We have discovered a novel disease of *Morella californica* and shown that it is caused by a species of the oomycete genus *Phytophthora*. Isolates of *Phytophthora* sp. from *M. californica* share some morphological and molecular similarities to *P. syringae*, but also differ from the latter in some regards placing its exact identity in doubt. Additional studies to clarify the identity of the organism are ongoing.

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