Research

Infection of Select Pinaceae and Cupressaceae Seedlings to *Phytophthora pinifolia*

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Abstract

Phytophthora pinifolia caused a devastating disease on Monterey pine (*Pinus radiata*) in Chile. This pathogen has not been reported in the United States, but there is concern should it arrive. There is little information regarding other hosts besides Monterey pine that may be susceptible to this pathogen. In the present study, other potential hosts within the Pinaceae and Cupressaceae were inoculated with zoospores of *P. pinifolia* and observed for symptoms and infection after 4 weeks. Similar to Monterey pine, knobcone (*Pinus attenuata*), bishop (*P. muricata*), and ponderosa

(*P. ponderosa*) pines were highly infectious, whereas several important species such as loblolly pine (*P. taeda*) and Douglas fir (*Pseudotsuga menziesii*) showed no infection. *P. pinifolia* was also not recovered from slash (*Pinus elliotii*) and eastern white (*P. strobus*) pines. This study is important because it demonstrates other economically important tree hosts are at risk by *P. pinifolia*, should it enter the United States.

Keywords: plant pathology, forestry, trees, potential hosts

Phytophthora spp. are known to cause some devastating diseases in natural forests worldwide including *P. ramorum*, *P. cinnamomi*, and *P. lateralis* (Hansen 2015). Several species have only been isolated from specific geographic regions, so their widespread impact is not fully understood. For example, *P. austrocedri* was first described in 2007 affecting a cypress forest in Argentina (Greslebin et al. 2007) and later junipers in Britain (Green et al. 2015). Another similar example is *P. pinifolia*, which was isolated from *Pinus radiata* (Monterey pine) in Chile in 2004, causing widespread death in pine plantations (Durán et al. 2008). In 2006, the disease affected approximately 60,000 ha of Monterey pine plantations in Chile. However, by 2008 the affected area was reduced to less than 500 ha (Durán et al. 2010) and remains low without causing significant defoliation (Hansen 2015). It is unknown why there was a reduction in the diseased area and appears to have occurred by some unknown natural phenomenon. Despite this decline in affected area and isolation of the pathogen only in Chile, the U.K. Forest Research has identified *P. pinifolia* as one of four high-risk damage agents (Anonymous 2013). USDA/APHIS has also listed this pathogen in the top five *Phytophthora* spp. of concern to the United States (G. Abad, personal communication). It is important to study and

characterize these pathogens specifically so that management decisions can be made should they appear in a new region.

Monterey pine, the primary host of *P. pinifolia*, is native to the California coast and nearby islands off Baja California (Rogers 2002). Monterey pine is important outside of California as well and is the most widely planted pine in the world (Deghi et al. 1995; McDonald and Laacke 1990). Besides the obvious impact to Monterey pine, the potential impact on other hosts, especially within the Pinaceae, is relatively unknown should this pathogen arrive in the United States. Included in their study, Ahumada et al. (2013) tested 13 *Pinus* species and varieties by wound inoculation on the stems. However, most of these species and varieties were native to Mexico and Central America, not commonly found in U.S. forests or plantations. In the United States, Southern plantation forests are mainly composed of pines, with an overwhelming majority (86.2% or 15.9 million ha) planted to the fast-growing loblolly (*Pinus taeda*) and slash (*Pinus elliotii*) pines (Zhang and Polyakov 2010). U.S. softwood lumber exports were valued at \$1.3 billion in 2014 (USDA-FAS 2015), and the total value of softwood stumpage sold from U.S. National Forests in 2013 was \$64.8 million (Howard and Jones 2016). Pines also play an important role in the ecosystem by improving habitat quality for many wildlife species, climate amelioration, soil stabilization, nutrient cycling, and serving as a reservoir for genetic material (Brockway et al. 2005). For example, in Southern U.S. forests, which is defined as the 13 states from Texas to Virginia, pines occupy 34% of forest lands (Wear and Gries 2012). The purpose of this study was to gain an understanding on the potential effects of *P. pinifolia* introduction on the U.S. pine resources to better evaluate the pathogen's host range and help fill in gaps related to risk maps and worldwide regulatory concerns.

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Plant Material

The species tested in this study were included because of their economic importance, seed availability for the study, and seed germination. Seeds of *Chamaecyparis lawsoniana*, *Larix laricina*,

Picea glauca, *Pinus attenuata*, *Pinus elliotii*, *Pinus muricata*, *Pinus ponderosa*, *Pinus radiata*, *Pinus rigida*, *Pinus strobus*, *Pinus taeda*, *Pseudotsuga menziesii*, *Sequoiadendron giganteum*, and *Thuja occidentalis* (Table 1) were planted in MT38 (7.6 × 20.3 cm; 950 cm³ volume) Mini-Treepots (Stuewe and Sons, Tangent, OR) containing a soilless potting mix (41% peat, 11% bark, 23% perlite, 23% vermiculite, 2% sand, and a small amount [0.35 liter per 500-liter volume] of granular trace element blend [Micromax Micro-nutrients, Everris NA]) and placed on a greenhouse bench under overhead irrigation. The plants were used when they were 6 to 12 months old and selected based upon uniformity in size.

Culture and Inoculation Preparation

A *P. pinifolia* culture (CBS122924), which was an ex-holotype isolate, was received from the Centraalbureau voor Schimmelf-cultures culture collection (Utrecht, the Netherlands). The culture was maintained for use on carrot juice agar at 25°C and on rye seed in sterile water in an incubator in the dark for long-term storage. The working culture was regularly reisolated from infected Monterey pine tissue (approximately after every third inoculation experiment) to maintain virulence.

Carrot juice was prepared by a modification of a method described by Erwin and Ribeiro (1996). Frozen diced carrots (200 g) were autoclaved in water (500 ml) for 20 min at 121°C, 1.4 bar and, once cooled, blended until liquefied. The volume was brought up to 1 liter with water. Carrot juice agar was prepared by adding 18 g of agar to 1 liter of the prepared carrot juice and autoclaving 20 min at 121°C, 1.4 bar. Clarified carrot broth was prepared by centrifuging the carrot juice for 20 min at 3,210 × g and carefully decanting the supernatant. The supernatant was then autoclaved prior to use.

Zoospores were produced by a method described by Widmer and Dodge (2015). Nine plugs (5-mm-diameter) were removed from the edge of a culture of *P. pinifolia* grown on carrot juice agar for approximately 8 weeks at 25°C in an incubator in the dark. Three plugs at one time were first ground into suspension aseptically with a pestle in a 1.6-ml Eppendorf microcentrifuge tube containing 0.5 ml of clarified 20% carrot broth. Afterward, the suspension was aseptically drawn out of the tube and transferred to Petri plates (90 mm diameter), each containing 15 ml of clarified 20% carrot

broth. The process was repeated until nine plugs in total were prepared as described above. The Petri plates were sealed by wrapping with plastic wrap and placed in a 25°C incubator in the dark. After 6 days, the cultures were rinsed three times with 0.1 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer, pH 6.2 (herein referred to as MES buffer). The cultures in the final MES buffer rinse were placed in a 20°C incubator under continuous fluorescent lighting (approximately 3,000 Lux) with the Petri plates not sealed. The following day, released zoospores were separated from the mycelium by gently drawing out the liquid with a pipette and filtering through a 53-μm mesh screen. The overwhelming majority of zoospores were motile at this time. The concentration of zoospores in the suspension was determined after vortexing a sub-sample to induce encystment so they could be counted on a hemacytometer.

Plant Inoculations

Just prior to inoculation, the needles of the seedlings were trimmed back with scissors as described by Widmer and Dodge (2015). The number of seedlings of each species per replication ranged between four and eight, depending upon the number of available seedlings at the time of inoculation. The foliage was sprayed with a zoospore suspension (50,000 zoospores/ml; 25 ml per eight plants with a DeVilbiss Number 15 hand pump atomizer [The DeVilbiss Company, Somerset, PA]). For controls, up to three seedlings of each species were inoculated only with MES buffer. The plants were immediately placed in a dew chamber with constant dew at 20°C for 1 week. Afterward, the plants were removed and placed on a greenhouse bench and watered daily with overhead irrigation. Temperatures in the greenhouse generally ranged from 25 to 30°C. Symptoms were monitored over time. After 4 weeks from inoculation, the plants were cut at the soil line, surface sterilized for 1 min in 70% ethanol, and rinsed three times for 10 min each in sterile water. The needles were removed from the stems, and the resulting stems and crowns (i.e., growing point of the stem) along with a random selection of removed needles were plated on a *Phytophthora* selective agar medium (PARPH+V8; Ferguson and Jeffers 1999) to verify infection. Three replicated experiments were conducted for each species, except *Larix laricina*, *Picea glauca*, *Pinus rigida*, and *Thuja*

TABLE 1
A list of plant species and their common names used in this study with the source of seed material

Plant species (family)	Common name	Seed source
<i>Chamaecyparis lawsoniana</i> (Cupressaceae)	Port Orford cedar	Forest Service, Dorena Genetic Resource Center, Cottage Grove, OR
<i>Sequoiadendron giganteum</i> (Cupressaceae)	Giant sequoia	Cal Fire, L.A. Moran Reforestation Center, Davis, CA
<i>Thuja occidentalis</i> (Cupressaceae)	Northern white cedar	Jardin botanique de Montréal, Montréal, Québec
<i>Larix laricina</i> (Pinaceae)	American larch	Jardin botanique de Montréal, Montréal, Québec
<i>Picea glauca</i> (Pinaceae)	White spruce	Jardin botanique de Montréal, Montréal, Québec
<i>Pinus radiata</i> (Pinaceae)	Monterey pine	Cal Fire, L.A. Moran Reforestation Center, Davis, CA
<i>Pinus attenuata</i> (Pinaceae)	Knobcone pine	Cal Fire, L.A. Moran Reforestation Center, Davis, CA
<i>Pinus muricata</i> (Pinaceae)	Bishop pine	Cal Fire, L.A. Moran Reforestation Center, Davis, CA
<i>Pinus ponderosa</i> (Pinaceae)	Ponderosa pine	Cal Fire, L.A. Moran Reforestation Center, Davis, CA
<i>Pinus taeda</i> (Pinaceae)	Loblolly pine	Forest Service, National Seed Laboratory, Dry Branch, GA
<i>Pinus rigida</i> (Pinaceae)	Pitch pine	Jardin botanique de Montréal, Montréal, Québec
<i>Pinus strobus</i> (Pinaceae)	Eastern white pine	Forest Service, National Seed Laboratory, Dry Branch, GA
<i>Pinus elliotii</i> (Pinaceae)	Slash pine	Forest Service, National Seed Laboratory, Dry Branch, GA
<i>Pseudotsuga menziesii</i> (Pinaceae)	Douglas fir	Cal Fire, L.A. Moran Reforestation Center, Davis, CA

occidentalis, which were replicated only two times, owing to limited plant availability.

Statistical Analyses

Individual seedlings were rated as symptomatic if they died, showed necrosis, or wilted. A seedling was rated as positive for *P.*

pinifolia if any colonies visually identified as *P. pinifolia* were observed on the selective medium growing from the plant tissue. The average percentage of symptomatic or infected seedlings was calculated based upon the total number of inoculated seedlings for each of the replicated tests. Owing to the variability between the replications, the percentage of symptomatic seedlings and the

FIGURE 1
Examples of symptom expression on Monterey pine (*Pinus radiata*) seedlings typically observed on all tested species, ranging from healthy control (A) to crown necrosis (B) to tip death (C) to complete death (D) 4 weeks after inoculation with *Phytophthora pinifolia* zoospores.

TABLE 2 Average percentage (± standard error) of seedlings that displayed necrotic symptoms and for which a <i>Phytophthora pinifolia</i> culture was recovered from plant material after plating on <i>Phytophthora</i> selective medium 4 weeks after inoculation with <i>P. pinifolia</i> zoospores				
Plant species	Common name	n ^y	Symptomatic seedlings (%)	Recovery of <i>P. pinifolia</i> (%)
<i>Chamaecyparis lawsoniana</i>	Port Orford cedar	23**	0.0 ± 0.0 e ^z	21.0 ± 10.5 b
<i>Sequoiadendron giganteum</i>	Giant sequoia	24**	0.0 ± 0.0 e	8.3 ± 8.3 bc
<i>Thuja occidentalis</i>	Northern white cedar	8*	12.5 ± 12.5 de	25.0 ± 0.0 b
<i>Larix laricina</i>	American larch	8*	25.0 ± 0.0 cd	25.0 ± 0.0 b
<i>Picea glauca</i>	White spruce	12*	0.0 ± 0.0 e	0.0 ± 0.0 c
<i>Pinus radiata</i>	Monterey pine	31**	67.4 ± 11.2 ab	57.6 ± 11.3 a
<i>Pinus attenuata</i>	Knobcone pine	26**	91.7 ± 5.0 a	87.5 ± 5.0 a
<i>Pinus muricata</i>	Bishop pine	24**	50.0 ± 7.2 bc	50.0 ± 7.2 a
<i>Pinus ponderosa</i>	Ponderosa pine	24**	50.0 ± 7.2 bc	62.5 ± 7.2 a
<i>Pinus taeda</i>	Loblolly pine	24**	0.0 ± 0.0 e	0.0 ± 0.0 c
<i>Pinus rigida</i>	Pitch pine	15*	18.8 ± 18.8 de	12.5 ± 12.5 bc
<i>Pinus strobus</i>	Eastern white pine	24**	0.0 ± 0.0 e	0.0 ± 0.0 c
<i>Pinus elliotii</i>	Slash pine	24**	0.0 ± 0.0 e	0.0 ± 0.0 c
<i>Pseudotsuga menziesii</i>	Douglas fir	23**	0.0 ± 0.0 e	0.0 ± 0.0 c

^y Total number of plants tested over two (*) or three (**) replications.
^z Values followed by the same letter within a column are not significantly different (*P* > 0.05) according to Duncan’s analysis after PROC RANKS in SAS for Windows (version 9.2; SAS Institute, Cary, NC).

percentage of seedlings infected with *P. pinifolia* were analyzed separately by using PROC RANKS and conducting an analysis of variance on the ranks in SAS for Windows (version 9.2; SAS Institute, Cary, NC). Comparisons among the tested species were done by subjecting the analysis of variance to Duncan's analysis in SAS for Windows.

Susceptibility to *P. pinifolia*

Results showed a range of symptom expression and *P. pinifolia* recovery among species within the Pinaceae and Cupressaceae (Table 2). Typical symptoms after 4 weeks ranged from complete necrosis and seedling death to crown necrosis (Fig. 1). No symptoms were observed or *P. pinifolia* recovered from white spruce, loblolly pine, eastern white pine, and Douglas fir (Table 2), as well as the controls (data not shown). Monterey pine and knobcone pine had the highest percentage of plants that were symptomatic. Bishop pine was not significantly different than Monterey pine in the percentage of seedlings that displayed symptoms and knobcone pine in the percentage of seedlings from which *P. pinifolia* was recovered (Table 2). This similarity is not surprising because knobcone and Monterey pine are closely related (Varelides 1995), and bishop pine is a hybrid of those two species (Critchfield 1967). All three species have the same natural distribution along the California coast (Varelides 1995). The worldwide importance of Monterey pine has been well-documented for its lumber and paper value (Bayne 2015). Knobcone pine is often used in rehabilitation projects after severe wildfires and is widely distributed from southern Oregon to Baja California (Fry and Stephens 2013). Planting bishop pine near its native range is also of interest because it is more resistant to red band needle blight (*Dothistroma pini*) than Monterey pine and has good growth and wood quality comparable to Douglas fir (Millar 1987). Because these three species are so closely related and react in a similar manner to *P. pinifolia*, there might be a common genetic factor such as susceptibility genes.

Another important susceptible species to infection is ponderosa pine, which is one of the most wide-ranging pines in North America (Shinneman et al. 2016). Ponderosa pine is economically an important species that is used for construction of buildings. Annual production of ponderosa pine lumber ranks third behind Douglas fir and hem-fir (Wennerberg 2004). If *P. pinifolia* were to enter a conducive environment with ponderosa pines, effects could be devastating not only economically but to wildlife habitat as well (Moir et al. 1997).

Three other species, northern white cedar, pitch pine, and American larch (tamarack), were also symptomatic but less than those mentioned above. Infection of northern white cedar is significant because this would be the first report of *P. pinifolia* infecting a host outside of the Pinaceae. Northern white cedar is an important conifer in the Great Lakes region and New England states of the United States and southern Canada for wildlife habitat (Miller 1990). It is slow to regenerate because its reproduction is limited after harvesting (Larouche et al. 2011). American larch has also been reported to be susceptible to *P. ramorum*, although not as highly as other species tested (Jinek et al. 2011). Pitch pine is a major component of forest types in the northeastern United States (Gucker 2007) and historically was an important tree for shipbuilding and other constructions because the wood resists decay. Loss of pitch pine habitat would be a concern owing to development and lack of natural regeneration (Hawley 2002).

Several species (Monterey pine, knobcone pine, and pitch pine) displayed a higher percentage of symptoms than recovery of *P. pinifolia* from the tissue (Table 2). As observed by Widmer and Dodge (2015), *P. pinifolia* was not always recovered in symptomatic plants that were completely dead. It appears that *P. pinifolia* survives mainly in living tissue or where necrosis is not advanced, as was observed with *P. alni* (Streito et al. 2002). To confirm this assumption, samples would need to be collected as symptoms first develop before the entire seedling becomes necrotic.

Loblolly pine, slash pine, and Douglas fir are three economically important species to the United States that were not susceptible to *P. pinifolia* infection at the seedling stage under the experimental conditions. Loblolly and slash pine are two endemic pine species in the southeastern United States that cover an area that is planted and commercially managed. Loblolly pine is present on more than 12 million ha across 14 states (Coyle et al. 2015). Slash pine is a fast-growing species that has been planted on more than 4.2 million ha, generating around \$16.3 billion in revenue for the State of Florida alone (Susaeta et al. 2016). Douglas fir is considered one of the world's most important trees that is valued for its lumber, Christmas tree production, and habitat for wildlife (Anonymous 2002). Results from this study are significant in finding that these species are not infected by *P. pinifolia* and confirm the results from Ahumada et al. (2013) that loblolly pine is not susceptible.

Eastern white pine and white spruce also showed no symptoms or *P. pinifolia* recovery from inoculated seedlings. Eastern white pine is valued for its use in quality construction and fine woodworking and is favored for its use in moldings and furniture. Eastern white pine has a fairly wide range of habitat that includes New England states, the Great Lakes region, and along the Appalachian Mountains to northern Georgia (Abrams 2001). White spruce is harvested for pulp and construction and is the northernmost tree species in North America. However, there were few white spruce seedlings inoculated owing to low seed germination, and this species should be retested to confirm these results.

Giant sequoias are well known for their massive size and age, whereas Port Orford cedar is valued for its horticultural uses and quality of wood. Both species are native to western United States, and giant sequoias are mainly grown in protected habitats. Both giant sequoia and Port Orford cedar showed no obvious symptoms after 4 weeks from inoculation, but *P. pinifolia* was recovered from 8.3 and 21.0% of the seedlings, respectively (Table 2). Further studies would need to be conducted to determine whether *P. pinifolia* is a true pathogen on these hosts and if symptoms would have been observed if kept for longer than 4 weeks. It is also possible that *P. pinifolia* could exist as an endophyte in these species. This has been observed in other *Phytophthora* spp. and host relationships. For example, *P. cinnamomi* was isolated from different native plant species in the jarrah forest that were asymptomatic and previously not considered to be hosts (Crone et al. 2013), and it infects *Zea mays* without expressing symptoms (Wetherbee et al. 1985). This endophytic relationship could cause regulatory concern for the potential spread of *P. pinifolia* by going undetected on asymptomatic plants.

Conclusions

Previously, only limited pine species not commonly found in the United States were tested for their susceptibility to *P. pinifolia*

(Ahumada et al. 2013). The results presented in this study along with those by Ahumada et al. (2013) demonstrate that susceptible pines exist throughout Central America, Mexico, and the United States that could be a pathway for introduction into the United States. The pines tested in the present study were primarily selected because of their economic and environmental value in the United States. Results of this study show that there is a difference in symptomatic expression and recovery of *P. pinifolia* within the Pinaceae and Cupressaceae. One of the most important pine species in North America, ponderosa pine, is susceptible, whereas another economically important species, loblolly pine, showed no symptoms or infection in this study. Results in this study also verified the susceptibility of Monterey pine (Ahumada et al. 2013; Widmer and Dodge 2015) and demonstrated closely related species were also susceptible under the experimental conditions. Risk maps identifying potential establishment of plant pathogens are important, but currently not enough data have been generated to publish one for *P. pinifolia*. Although inoculations were done under artificial conditions in this study, the data will be useful in risk map development, as similar studies have shown for other pathogens such as *P. ramorum* (Hansen et al. 2005; Koch and Smith 2008). However, other epidemiological factors such as climatic suitability also need to be considered. For example, *P. pinifolia* cultures can grow in a temperature range of 10 to 30°C (Durán et al. 2008) and infect Monterey pine between 15 and 25°C (Widmer and Dodge 2015), but there are no data for infection outside of those ranges. Therefore, temperature may limit the actual impact on some northern species, such as pitch pine or Northern white cedar, where sufficient pathogen populations may not be able to establish to high enough levels because of the low winter temperatures. However, the results in this study will be beneficial along with more ecological studies in the development of a risk map for a pathogen that is of important worldwide regulatory concern.

Acknowledgments

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