

Methods for Screening Port-Orford-Cedar for Resistance to *Phytophthora lateralis*

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Abstract

Port-Orford-cedar (*Chamaecyparis lawsoniana* (A. Murray) Parl.) (POC) is an economically and ecologically valuable tree in the forests of southwest Oregon and northern California and in the horticultural trade worldwide. *Phytophthora lateralis*, the aggressive, invasive cause of POC root disease, was introduced to the native range of POC about 1950, and has since killed trees along roads and streams throughout the forest area where the tree grows. The federal forest management agencies responded with an integrated disease management program designed to slow further spread of the pathogen in order to protect remaining POC and restore the species with planted resistant stock in impacted areas. Various short-term resistance screening methods have been used since the 1980s. Ultimately, in any resistance program, the screening assays have to be ground truthed using field trials and the screening methods updated and refined to assist in resistance evaluations. In this paper we describe and compare the various inoculation methods and screening tests we use to evaluate POC parents and their progenies for resistance and offer evolving insights on their utility. This should provide food for thought for other newer resistance programs working with other pathogens or insects.

Introduction

Port-Orford-cedar (*Chamaecyparis lawsoniana* (A. Murray) Parl.) (POC) is an economically and ecologically valuable tree in the forests of southwest Oregon and northern California and in the horticultural trade worldwide. *Phytophthora lateralis*, an exotic, invasive root pathogen, is the primary cause of POC mortality in southwest Oregon and northwest California (Betlejewski et al. 2011). The origin of *P. lateralis* is not known, although it was first described killing POC in horticultural nurseries in the 1920s (Hansen et al. 2000). Recently *P. lateralis* or a very close relative was reported from native *Chamaecyparis obtusa* (Siebold & Zucc.) Siebold & Zucc. ex Endl. forests in Taiwan (Brasier et al. 2010), suggesting that the pathogen originated in eastern Asia. In North America it spreads as sporangia and zoospores in streams and is transported by vehicles along roads in the forest (Hansen et al. 2000). Even in infested areas, however, occasional Port-Orford-cedars escape mortality by *P. lateralis*.

Phytophthora lateralis was introduced to the forest range of POC about 1950. It has since killed trees along roads and streams throughout the tree's range. The federal forest management agencies—U.S. Department of Agriculture, Forest Service (USFS) and U.S. Department of the Interior, Bureau of Land Management (BLM)—have responded with a disease management program integrated into forest plans (Betlejewski et al. 2003, USDA FS and USDI BLM 2004). Mandated actions are designed to slow further spread of the pathogen in order to protect remaining POC, and to restore the species with planted resistant stock in areas where the pathogen has eliminated POC. Since the late 1980s, the USFS and the BLM, in cooperation with Oregon State University (OSU), have conducted an intensive program to identify and test resistant trees from the field and propagate them in a seed orchard with the goal of providing resistant seedlings for regeneration (<http://www.fs.fed.us/r6/dorena/poc/>; Snieszko 2006, Snieszko et al. 2006; Snieszko et al., Operational program to develop *Phytophthora lateralis* resistant populations of Port-Orford-cedar (*Chamaecyparis lawsoniana*), these proceedings). In this paper we describe the various inoculation methods and screening tests we use to evaluate POC parents for resistance.

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Most POC trees in the field are very susceptible, but genetic resistance to this pathogen has been demonstrated (Hansen et al. 1989, Snieszko 2006, Snieszko et al. 2006). Susceptible seedling families showed only 0 to 10 percent survival using a variety of inoculation techniques. Rooted cuttings of the most resistant parents were seldom killed, and seedling families of these parents exhibited 25 to 100 percent survival, depending on family and inoculation technique (Oh et al. 2006; Snieszko et al., Nine year survival of 16 *Phytophthora lateralis* resistant and susceptible Port-Orford-cedar families in a southern Oregon field trial, these proceedings). Symptom development on resistant trees, including reduced colonization and reisolation success, sunken lesions, and resinosis, was consistent with a hypersensitive reaction (Oh and Hansen 2007).

Methods

Screening Protocol

The USFS and BLM funded and soon led the effort to locate candidate resistant trees in the forest and to collect vegetative material and seeds for testing. Inoculations and resistance screening carried out at OSU have evolved through time to take advantage of the unique features of POC and to meet the changing needs of the program. Candidate parents from the field are initially challenged with a rapid stem dip zoospore inoculation test. “Winners” are then propagated as rooted cuttings, which are in turn exposed to zoospore inoculum in a root dip test. The best individuals are incorporated into containerized seed orchards at Dorena Genetic Resource Center (DGRC) in Cottage Grove, Oregon. Seedlings from cross or self pollination within the orchards are further challenged with the root dip test and by planting into infested soil for a 2-year exposure in outdoor raised beds. Finally, the most promising materials are included in long-term field tests on infested sites and maintained in the seed orchards by breeding zone. Over 10,000 field selections have been screened and over 1,100 trees have been chosen for further evaluation (Snieszko et al., Operational program to develop *Phytophthora lateralis* resistance populations of Port-Orford-cedar (*Chamaecyparis lawsoniana*), these proceedings).

Inoculum Production

Port-Orford-cedar cuttings, rooted cuttings, and seedlings are inoculated with zoospores. Two isolates of *P. lateralis*, collected from diseased POC trees in southern Oregon or northern California, were used. Isolates were grown on homemade corn meal agar amended with *B*-sitosterol (CMAB) (15 g Bacto agar, 50 ml corn steep, 20 mg *B* -sitosterol). Corn steep was made by mixing 100 g yellow corn meal in 650 ml de-ionized water at 60 °C for 1 hr. Solids were removed by pouring through eight layers of cheesecloth then filtering through Celite 545. Cultures were maintained in water storage in the culture collection at Oregon State University.

To produce zoospores, isolates were grown for one week on CMAB then three, 3 mm dia. plugs cut from the advancing edge of colonies were transferred to pea broth (150 g dry split peas per liter, autoclaved for 3 min and strained through a double layer of cheesecloth, then autoclaved for 60 min at 121 °C) and grown for 7 days. The culture medium was removed, plates were rinsed with de-ionized water, then filled with 10 ml filtered water from a local stream. After 24 hours, zoospore release was triggered, if needed, by chilling plates 1 hr at 5 °C then returning to room temperature.

Stem Dip Test

Initial screening of POC trees for resistance to *P. lateralis* is done on fresh cuttings from candidate trees. Six to 10 branch tips, each about 30 cm long, were clipped from each candidate tree, labeled, and transported cool and moist to the OSU lab for testing.

For zoospore inoculations, zoospores were produced as described above, and the water containing the colonies and zoospores was poured from plates into 1 L glass jars. Water and zoospores from one plate of each isolate, along with additional water to total 40 ml, were combined in each jar to an approximate depth of 1 cm.

Branches were freshly trimmed to 20 to 30 cm length, mixed among the candidate and control trees in two replications (three to five branches per parent per replication), and clustered in bundles of 10 then placed cut end down in each glass jar with zoospores, care being taken to insure that each cut end was immersed (fig. 1).

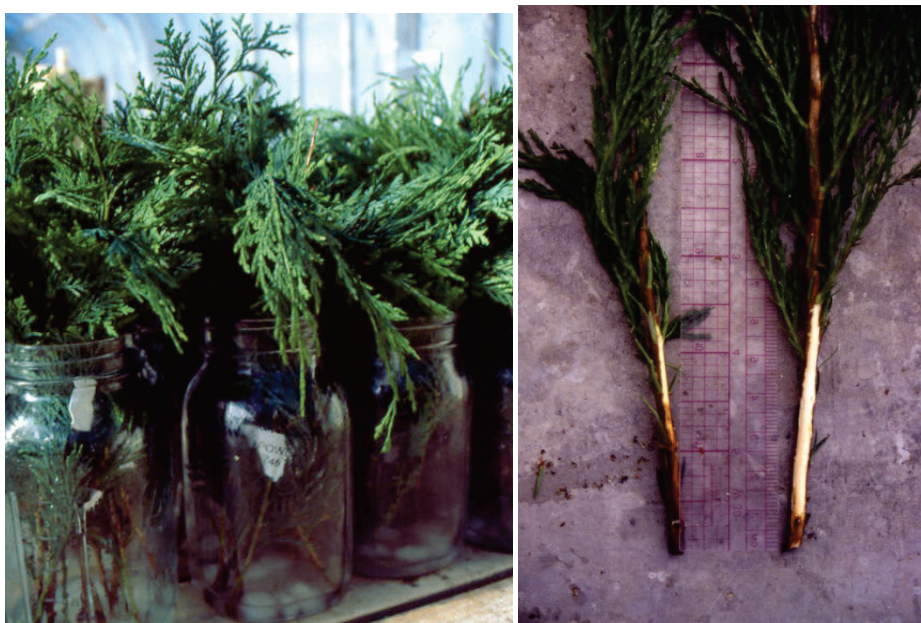


Figure 1—Stem dip test. Left: Branch tips with cut ends immersed in zoospore suspension. Right: Lesions on susceptible control (left) and resistant control (right) branches after three weeks.

Branches remain with cut ends in the zoospore suspension for 24 hours and then are transferred individually to plastic growth tubes filled with wet vermiculite. Racks of test cuttings are held in the greenhouse and watered regularly. After 3 weeks, they are scored. Each branch tip was removed from the vermiculite, rinsed, and scraped at the cut end to reveal the discolored lesion in the inner bark. Lesion length was recorded. The best 10 percent of all parent trees in each test, including all trees with average lesion lengths less than or equal to the resistant control, were declared “winners” and advanced to further testing.

Root Dip Test

Port-Orford-cedar for root dip inoculation trials was grown from cuttings from “winner” parent trees. Cuttings were rooted under mist and grown about 12 months. Rooted cuttings were grown in individual 3.7 cm top diameter, 20.6 cm length, ‘supercell’ plastic conical tubes (Cone-tainer™). Seedlings, mostly from controlled crosses at DGRC, were grown for about 9 months in supercells. Seedlings or rooted cuttings were grown until roots emerged from the bottom of the containers. Emergent roots were trimmed immediately prior to inoculation.

Seedlings or rooted cuttings were randomized and groups of six were clustered in each inoculation container. The lower 1 cm of the root system (in the growth tube) was immersed in a *P. lateralis* zoospore suspension (prepared as above) for 24 hours (fig. 2). The inoculated trees were then incubated in the greenhouse for up to 36 months, and symptom development was periodically recorded. At the first sign of yellowing or wilting (usually after about 6 months), a small slice in the bark at soil level was made and the stem examined for the characteristic necrotic lesion caused by *P. lateralis*. Mortality was recorded when the lesion reached the top of the growth tube. Mortality, evidenced by diagnostic lesion at the ground line, was noted regularly and the days from planting to mortality recorded.



Figure 2—Root dip test. Container grown seedlings and rooted cuttings, inoculated by immersing the bottom 1 cm of the container in zoospore suspension.

Port-Orford-cedar seedlings and rooted cuttings were also planted in infested soil in an outdoor raised bed (fig. 3). Two raised beds were established in 2000, each 1.5 x 10 m and 30 cm deep, and contained a soil mix comprised of 1/4 loam, 1/4 peat, 1/4 pumice, 1/4 fine bark, plus 1/2 kg gypsum/m³, and 1 kg lime/m³. The soil mix was infested with *P. lateralis* by mixing sporulating cultures and chopped roots of infected POC seedlings with the soil. Port-Orford-cedar trees were planted in family row plots in a replicated design and received regular summer irrigation. Mortality, evidenced by diagnostic lesion at the ground line, was noted regularly and the days from planting to mortality recorded.



Figure 3—Raised bed test. Seedlings and rooted cuttings planted in infested soil.

Outplanting Tests

Port-Orford-cedar seedlings and rooted cuttings were also planted in artificially or naturally infested soil at field sites. Some of the first selected families and clones were planted in 1989 and 1990 in a replicated block design at the OSU Botany and Plant Pathology Field Lab near Corvallis, Oregon (table 1). Parent trees other than parent id numbers CF1 and ESOC were initially collected by Professor L. F. Roth from forested areas in Coos County, Oregon, either because they were survivors in areas of widespread mortality or as unselected control trees from areas with no previous mortality. The 1/10 ha plot had been artificially infested with *P. lateral* some years previously, and was initially used for screening different species of *Chamaecyparis* for resistance to *P. lateral*. Mortality and cause of death were recorded annually to the present. Surviving trees are now 10 to 20 cm dbh. Additional long-term outplanting test sites were selected at forest sites where local concentrations of POC had been recently killed by *P. lateral* (fig. 4).

Table 1—Survival of POC trees selected for resistance or as susceptible controls at the Oregon State University Botany Farm outplanting site

Parent tree	Year planted	Trees planted (n)	Trees alive July 2011 (%)
CF1-cuttings	1989	25	48.0
CF1-seedlings	1989	25	24.0
CF2	1989	20	80.0
CF3	1989	4	50.0
CF4	1989	20	15.0
ESOC	1990	30	3.3
CHECK10	1990	20	0.0
CB1	1989	20	40.0
CB2	1989	20	0.0
CB4	1989	18	5.6
CB5	1989	20	0.0
CHECK13	1989	20	0.0
CHECK14	1989	20	0.0



Figure 4—Forest outplanting site established on naturally infested ground within a mixed Douglas-fir-POC forest in Douglas County, Oregon.

Results

Snieszko et al. present extensive validation data for the screening tests, comparing greenhouse and raised bed rankings with outplanting test results elsewhere in this proceedings. Here we present two additional examples. The stem dip test reliably separated a small number of POC families with good survival at the Botany Farm planting from susceptible families with poor or no survival (table 2). Similarly, root dip test scores reliably predicted survival after 2 ½ years at an infested outplanting site (fig. 5).

Table 2—Stem dip lesion length scores for branch tips collected from susceptible and resistant trees from the Botany Farm outplanting site

Parent tree	Alive 2011 (%)	Stem dip score (mm)
Resistant control		9.2
CF1 (cuttings)	48	9.4
CF2	80	12.5
CB1	45	10.7
CB2	0	28.2
CB4	6	28.9
CB5	0	28.9
Susceptible control		32.7

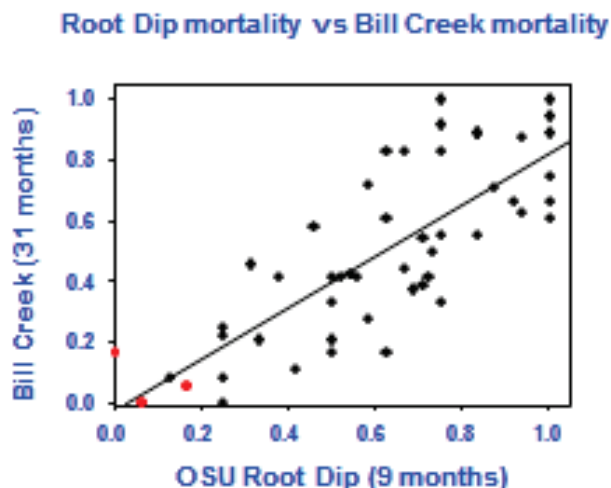


Figure 5—Root dip vs outplanting survival, Bill Creek test site. See Snieszko et al. 2006 for trial background.

Discussion

Short-term assays of resistance are a vital part of operational resistance programs. The four methods of short-term screening currently used for POC to discern *P. lateralis* resistance—(1) stem dip; (2) root dip using rooted cuttings; (3) root dip using seedlings; and (4) raised bed—all have potential advantages. The stem dip test can be done fastest and cheapest. The raised bed trial may mimic field conditions or could be monitored for three years or more for continued mortality or evolution of virulence. The root dip tests may allow evaluation of hundreds of families or clones at a time and provide very controlled conditions. However, ultimately the utility of these tests depends on how their results relate to field trials. At this point in time, the seedling root dip assay, and probably the raised bed trials, provide the best overall indication that there might be several types of resistance and

information on the upper level of survival to expect under field conditions (Snieszko et al., Nine year survival of 16 *Phytophthora lateralis* resistant and susceptible Port-Orford-cedar families in a southern Oregon field trial, these proceedings; Snieszko and Savin, unpublished).

The POC resistance screening program is an evolving success. Resistant seed with demonstrated survival potential is available for regeneration programs in the most important POC seed zones. The program continues, screening additional forest trees as candidates are identified, and root dip testing a backlog of stem dip “winners” to add additional parents to the orchards. In addition, orchards are continuously rogued and upgraded as field testing results are available and through selection of resistant progeny from controlled crosses.

Each of the screening tests has advantages as well as limitations. The original intention of the stem dip test was to provide a quick first screen of candidate trees assuming most would prove susceptible and could be eliminated before costly propagation efforts are made. It is likely that such an indirect test can only select for one type of resistance. However, with the compilation of data from a large number of selections, a re-evaluation of the utility of the stem dip test is underway. The test identifies some of the parents with the highest and lowest levels of resistance, but has a low correlation with the root dip tests (Snieszko and Savin, unpublished). It is not sensitive enough to rank trees with intermediate levels of disease tolerance. There appear to be several types of resistance in POC (Snieszko 2006; Snieszko, unpublished), and more understanding of these mechanisms is needed to fully evaluate the limitations of the stem dip method. We hope to use other current existing data or selections to more fully investigate the utility of this method. Other ‘indirect’ methods (e.g., biochemical markers, or genetic markers) of selecting for different types of resistance could be useful in short-cutting the initial testing step.

Rankings from root dip testing correlate well with outplanting test results (Snieszko et al., Nine year survival of 16 *Phytophthora lateralis* resistant and susceptible Port-Orford-cedar families in a southern Oregon field trial, these proceedings). The infection pathway is more realistic and the longer test period allows at least preliminary identification of “slow to die” families. Seedlings outgrow their containers after 2 or 3 years, however, and rooted cuttings are often difficult to evaluate because they may be slow to develop a robust root system. In a recent comparison of rooting cuttings and seedling families, there was good agreement, but the seedling assay appeared more consistent (Snieszko and Savin, unpublished). The current seedling assay takes several years to complete; the seedlings are grown for 9 months and then followed for 1 to 3 years after inoculation. Now that we have well defined resistant and susceptible families, there may be merit to see if we can short-cut the process by utilizing much younger, smaller seedlings in smaller containers. If so, this could reduce some costs as well as speed evaluation.

The various outplanting trials, especially on high-hazard forest sites, provide the ultimate test of the program, but are also most difficult to establish and maintain. It is challenging to find sites of sufficient area with uniform inoculum and topography. Regular recording of mortality is often precluded by limited travel budgets, and other mortality agents confuse the analysis.

Lessons learned in the POC resistance program should be valuable in providing guidance to other programs. Blind faith in a single short-term seedling assay may lead to over- or under-estimation of the level and types of resistance or may preclude utilizing some types of resistance. Establishing some field trials early to help validate short-term screening methods is essential to helping maximize progress in the development of resistant populations. The development of new screening assays such as biomarkers or genetic markers could be a useful addition to any resistance program, provided they are validated with field trials.

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