Interactions Between Root Rotting *Phytophthora*, *Abies* Christmas Trees, and Environment

Kathleen M. McKeever^{1,†} and Gary A. Chastagner²

¹USDA Forest Service, Forest Health Protection, Southern Region, Asheville, NC 28806; and ²Department of Plant Pathology, Washington State University Puyallup Research and Extension Center, Puyallup, WA 98371

Abstract

Defining host-pathogen interactions between species of root-rotting *Phytophthora* and *Abies* in Christmas tree production areas is important for tailoring management activities on a regional scale and for developing molecular tools for identifying resistant host species. Classifying *Abies* species as resistant or susceptible is complicated by regional variation in abundance and aggressiveness of *Phytophthora* species and the influence of environment on symptom expression and host vigor. Because previous studies performed to assess host response to *Phytophthora* root rot (PRR) have focused on one or a few species of either the host or pathogen, a multifactorial experiment was conducted to assess the responses of seven species of *Abies* challenged with three isolates each of four *Phytophthora*

species under contrasting temperature conditions. Evaluation of mortality, root rot severity, and remaining root biomass after 16 weeks of exposure to the pathogen confirmed prior inferences regarding inherent variation in the resistance responses of various species of *Abies* and demonstrated evidence of variation in aggressiveness among species of *Phytophthora* as well as different isolates of the same *Phytophthora* species. The ambient temperatures at which studies were conducted had a conspicuous effect on host mortality, root rot severity, and radial growth of *Phytophthora*. Understanding how host responses differ under variable pathogen attack and ambient environment will improve efforts to control PRR using host species substitutions on infested ground.

Phytophthora root rot (PRR) is a serious disease of true fir (*Abies* spp.), resulting in substantial losses in the Christmas tree and conifer nursery industries (Chastagner and Benson 2000; McKeever and Chastagner 2016). Despite the availability of select chemical alternatives and cultural control methods, practical options for reducing PRR losses in Christmas tree farms are scarce, and their utilization may be limited by expense or feasibility of execution (Frampton and Benson 2012). Extreme susceptibility of Fraser (*Abies fraseri* [Pursh] Poir.) and noble (*A. procera* Rehder) firs often exceeds the practical efficacy of existing control measures available to Christmas tree farmers and has dictated grower abandonment of *Phytophthora*-infested land for cultivation of these highly desirable species (McKeever and Chastagner 2016). Increased demand by Christmas tree consumers for susceptible species, and the necessity for growers of live Christmas trees to remain relevant in competition with artificial tree industries, has spurred investigation into ways to reclaim infested acreage.

Recommending PRR-resistant *Abies* species and developing genomic tools to identify resistance to PRR are attractive mitigation options that rely on the ability to definitively assign a host resistance phenotype that is robust over a range of external influences. Host phenotype designation in the PRR pathosystem is fraught with complicating factors including variation in *Phytophthora* species aggressiveness and the fact that *Phytophthora* species compositions differ among U.S. Christmas tree-growing regions. Also, because climates differ in various parts of the country, factors such as heat and

moisture stress influence host vigor, pathogen communities, and thus potential resistance expression.

Studies that have been conducted to characterize *Abies* resistance to PRR illustrate a need for a comprehensive approach to unify multiple contributing factors into an experiment in which phenotype can be interpreted within a controlled context. A history of testing *Abies* seedlings under lathe house conditions by researchers at North Carolina State University using a North Carolina isolate of *P. cinnamomi* Rands has indicated little to no resistance to *P. cinnamomi* in North American firs but good survival of the exotic momi (*A. firma* Siebold & Zucc.), Himalayan (*A. pindrow* [Royle ex D. Don] Royle), Siberian (*A. sibirica* Ledeb.), and Turkish firs (*A. bornmuelleriana* Mattf.) (Frampton and Benson 2004, 2012; Hinesley et al. 2000; Shew and Benson 1981). Surveys of field-grown Turkish fir in northwestern North Carolina and northern California indicate that although seedlings of this species tolerate *Phytophthora* inoculation experimentally, they routinely succumb to *P. cinnamomi* in Christmas tree production areas where *P. cinnamomi* is pervasive (McKeever and Chastagner 2016). A field study in Washington state that evaluated 11 *Abies* spp. for root rot and stem cankers caused by *Phytophthora* spp. commonly found in Pacific Northwest bareroot nurseries corroborated findings regarding the aggressiveness of *P. cinnamomi* but also classified *P. cactorum* (Lebert & Cohn) J. Schröt., *P. cambivora* (Petri) Buisman, and *P. cryptogea* Pethybr. & Laff. as similarly virulent (Chastagner et al. 1990). *P. cambivora* was the most abundantly isolated species from field-grown noble and Fraser firs during collections from Christmas tree operations in western Washington and Oregon, suggesting its prevalence in these areas and virulence on these species (McKeever and Chastagner 2016). Experimental differences in disease development owing to isolate choice was illustrated on conifer seedlings for nine *Phytophthora* species in the Pacific Northwest (Hamm and Hansen 1982), and Campbell and Hamm (1989) supported the trends of variation in *Phytophthora* spp. aggressiveness even on non-*Abies* conifers. These research results suggest that to elucidate host phenotypes for *Abies* infected with *Phytophthora* across U.S. Christmas tree growing regions, it is necessary to design a study to incorporate combinations of host and pathogen species under conditions that reflect variations in regional climates.

In an effort to satisfy this need, a multifactorial greenhouse experiment was implemented in which seven *Abies* species were inoculated with three isolates of each of four *Phytophthora* species at

[†]Corresponding author: Kathleen M. McKeever;
E-mail: kmckeever@fs.fed.us

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two temperatures. The goals of this study were to (i) quantify root rot severity and mortality within an *Abies* species owing to inoculation with four different *Phytophthora* species, (ii) investigate how temperature affects the overall rates and sums of mortality, (iii) observe differences in root rot severity and mortality in response to infection by different isolates of a *Phytophthora* species, (iv) evaluate visual root rot severity ratings and rootball dry weights for their usefulness in predicting mortality, (v) relate radial growth of *Phytophthora* isolates in vitro to virulence in vivo, and (vi) interpret host resistance with respect to *Phytophthora* species and temperature to improve recommendations to Christmas tree growers about which *Abies* species will survive best under local environmental conditions and *Phytophthora* populations.

Materials and Methods

Experimental design. Four species of *Phytophthora* were used to challenge seven *Abies* spp. at two temperature ranges. A cool temperature range of 15 to 21°C (further referred to as 15°C) was chosen to simulate environmental conditions that may be encountered year-round in more temperate geographic locations such as the Pacific Northwest, or at cooler times of the growing season in other locations. A warm temperature range of 27 to 32°C (further referred to as 27°C) was chosen to suggest the typical summertime temperatures encountered in many U.S. Christmas tree growing regions. Assessing PRR at two different temperatures required that the experiment be arranged in two separate greenhouses. Because variation exists among isolates within a given *Phytophthora* species (Eggers et al. 2012; Granke et al. 2011), the experiment was partitioned into three simultaneously running experiments, each employing a different isolate of the four *Phytophthora* species. Five replicate blocks of each *Phytophthora/Abies* combination were executed at each temperature.

Each of the four species of *Phytophthora* plus one noninoculated control treatment were randomly assigned within each replicate block. Within each *Phytophthora* species subdivision, three geographically distinct isolates of that given *Phytophthora* were randomly distributed. Inoculum of each species × isolate treatment (or a noninoculated treatment) was applied to a set of seven different fir species. Each species of fir was made up of five individual seedlings representing an experimental unit, of which collected data points were averaged to generate a single measurement. Because the experimental units (composed of five trees) were replicated in each of five blocks, the blocks constituted five true replicates with measurements that were not averaged.

Seedlings. The seven species of *Abies* assessed in this study represent the most commonly grown and economically important Christmas tree species for U.S. markets. Five of the species are native to North America: Fraser, noble, white (*A. concolor* [Gordon] Lindley ex Hildebrand), Canaan (*A. balsamea* var. *phanerolepis* Fern.), and balsam firs (*A. balsamea* [L.] Mill.). The two remaining species used in this experiment, Turkish and Nordmann fir (*A. nordmanniana* [Steven] Spach), are native to the Caucasus Mountains spanning northern Turkey and western Eurasia and belong to the *A. nordmanniana* complex. Seedlings were obtained in February 2014 and 2015 from the Weyerhaeuser Corporation's Rochester Nursery (Rochester, WA). The five North American species were grown as 1-year-old containerized stock (P-0). Owing to the small size of P-0 Nordmann and Turkish fir seedlings, 2-year-old bareroot seedlings (P+1) of similar size to the P-0 stock of the other five species were used. All seedlings were received frozen and physiologically dormant. In June of each year, thawed seedlings were transplanted into 262-ml D16L Deepots (Stuewe & Sons, Tangent, OR) using a custom conifer potting mix prepared by Specialty Soils (Covington, WA) (Table 1). Planted seedlings were placed into D50T 50-cell-capacity support trays and maintained outdoors from June through October on elevated lumber-and-cinder benches underneath 50% shade cloth. Irrigation was initiated via overhead sprinkler two times daily for 20 min increments for the first 2 weeks during transplant establishment and subsequently thereafter during hot spells (>29°C). A regular outdoor watering schedule was maintained once daily with a 15-min duration during temperate weather. Two times during the

outdoor storage period, seedlings were treated with fludioxonil (Medallion, Syngenta Crop Protection, Greensboro, NC) applied as a spray until runoff at a rate of 56.7 g (2 oz) per 378.5 liters (100 gal) of water for the prevention of gray mold (*Botrytis cinerea* Pers.) during storage. In early October, as seedlings were entering dormancy following terminal bud development, the seedlings—still potted in the Deepots—were packaged in wax-coated seedling boxes to undergo an artificial vernalization period at 3°C in the dark for 800 h (approximately 4 weeks). This vernalization period allowed for experiments to be conducted on dormant seedlings and ensured consistent budbreak upon resumption of physiological activity. Upon termination of the vernalization period, seedlings were removed from the boxes, labeled with pull-through tree tags, and randomized into the two greenhouses where experiments were to be conducted. During the 3-week period between introduction into the greenhouses and initiation of the experiment, greenhouse conditions were kept at a cool temperature between 12 and 15°C with no artificial lighting and drawn shade cloth to allow for easy transition from cold-storage temperatures to the warm, lighted temperatures that would be implemented during the experiment. Irrigation during this transitional period was applied once daily for 10 min durations at a rate of 0.25 ml/min.

Selection of *Phytophthora* species and isolates. *Phytophthora* cultures used for inoculum in this study were sourced from a nationwide collection of root-rotting *Phytophthora* from *Abies* in Christmas tree farms conducted during a 2-year period from 2012 to 2013 (McKeever and Chastagner 2016). It was decided that the four most commonly occurring *Phytophthora* species from this survey would be selected to constitute the inoculum. These species included *P. cambivora* (11 isolates), *P. cinnamomi* (seven isolates), *P. kelmiana* (seven isolates), and *P. pini* Leonian (four isolates) (McKeever and Chastagner 2016). Because several isolates of each of the four *Phytophthora* species were collected, virulence testing was performed to acquire the three most aggressive isolates of each species to test concurrently in the planned greenhouse experiment. Table 2 provides information on the origins and NCBI identities of each isolate employed in the virulence assessment. Lesion lengths resulting from seedling stem inoculations were chosen to infer virulence, because this is an established method for *Phytophthora* that correlates well to aggressiveness on roots (Bhat et al. 2006; Weiland et al. 2010).

Prior to assessing virulence, all collected isolates of the four *Phytophthora* spp. of interest were inoculated onto noble fir branches in a preemptive effort to maximize wild-type virulence after repeated subculturing on artificial media (Hodgson and Sharma 1967). For

Table 1. Bulk custom soil mix prepared by Specialty Soils (Covington, WA) used to pot seedlings for all experiments

Quantity per m ²	Component
2%	Diatomaceous earth
27.84%	Saskatchewan sphagnum peat
17.5%	Pumice
5%	Nitrified alder sawdust
50%	Aged fine bark (hemlock and fir)
0.113 kg	M-ROOTS 3-3-3 (mycorrhizae endo/ecto)
0.005 kg	Treflan 10G
0.172 kg	Calcium nitrate (Ca[NO ₃] ₂) (15.5-0-0)
0.131 kg	Potassium sulfate (K ₂ SO ₄) (0-0-53)
0.127 kg	Ammophos (NH ₄ H ₂ PO ₄) (11-52-0)
0.376 kg	Ferrous sulfate (FeSO ₄) (20% Fe)
2.494 kg	Plantacote (14-9-15) + S (4-mo. release)
0.172 kg	Agricultural gypsum (CaSO ₄ 2H ₂ O)
1.700 kg	Limestone flour
2.721 kg	Dolomite lime
14.78 ml	Manganese sulfate (MnSO ₄) (2.5% Mn)
3.84 ml	Sodium molybdate (Na ₂ MoO ₄) (2.0% Mo)
29.57 ml	Fritted micronutrients
31.64 ml	TriCure media surfactant

restorative inoculations, all isolates of a single *Phytophthora* species were inoculated onto different branches of the same field-grown noble fir. Inoculations were made in the second year's internodal region of a branch by scoring the wood with a 5-mm-diameter cork borer and peeling back the thin bark layer to expose the phloem underneath. A 5-mm-diameter plug from an actively growing 5-day-old culture on V8 medium was applied facedown to the phloem, and the bark layer was replaced. Inoculation sites were sealed with sterile petroleum jelly, wrapped in moist sterile cotton, and fastened with Parafilm (Bemis, Neenah, WI). Inoculation sites were allowed to incubate on the tree for 14 days at ambient outdoor temperatures averaging 19°C (diurnal fluctuations $\pm$ 7°C) with 71% average relative humidity. After 14 days, samples were taken from the margins of the lesioned and healthy tissues surrounding the inoculation sites, surface sterilized with a 1:10 solution of 8.25% NaClO to sterile deionized water, and plated into PARPH-clarified V8 (cV8) semiselective medium (15 g of Difco agar, 33 ml of clarified V8 juice, 10 mg of Delvocid salt [50% pimaricin], 250 mg of sodium ampicillin, 10 mg of rifampicin sodium salt, 67 mg of Terracolor [75% pentachloronitrobenzene], and 50 mg of hymexazol in 1 liter water). Resulting cultures were transferred to unamended cV8 medium.

Virulence testing was performed on actively growing 2-year-old bareroot noble fir seedlings that had been transplanted into 1-liter MT38 Treepots (Stuewe & Sons) and maintained outdoors for 4 weeks prior to use. Virulence testing was performed in August 2014 in a randomized complete block design with three seedlings constituting an experimental unit and three replications (blocks) of each treatment. The experiment was repeated once. Height and diameter measurements were recorded for each seedling in an effort to choose uniform plants for inoculations. Inoculations were performed on the stem of the seedling approximately 12 cm above the soil line, and techniques were identical to the restorative isolations described above. Treatments were randomized within each block, and seedlings were incubated in a 17°C greenhouse for 7 days with shade

cloth drawn and no supplementary lighting. Overhead irrigation was applied for 10 min, once a day, at a rate of 0.25 ml/min. After 7 days, resulting lesions were exposed by carefully scraping back the bark starting at the point of inoculation and extending to the perimeters of the lesion. Lesion lengths were measured with digital calipers.

For the final 12 isolates (three isolates of each of the four species of *Phytophthora*) that were selected based on stem lesion lengths, radial growth measurements were assessed at two different temperatures corresponding to the two experimental temperatures. Because the cool temperature ranged from 15 to 21°C and the warm temperature ranged from 27 to 32°C in two separate greenhouses, incubators were set to the midpoint values of 17 and 29°C for the culture radial growth assessments. Plugs (4-mm diameter) of each isolate were grown on cV8 medium in three replicate Petri dishes. Radial growth (mm) in two opposing directions was recorded 24 h after plating and then daily for up to 8 days. Measurements for opposing directions of each plate and for the three replicate plates were averaged for each isolate. This assay was repeated once.

Inoculation. Colonized rice grains were used to apply experimental treatments to seedlings (Holmes and Benson 1994). For each treatment, 8 g of long-grain white rice were mixed with 5.76 ml of deionized water in a 250-ml flask, autoclaved twice, and seeded with six 3-mm-diameter plugs taken from the edges of 4-day-old cultures grown on cV8 medium. Noninoculated treatments received six plugs of noncolonized cV8. Flasks were incubated at room temperature for 21 days, with agitation on alternating days to break up rice mats and evenly distribute inoculum. A separate set of 15 flasks (three isolates each of four *Phytophthora* spp. and three noncolonized control flasks) was produced for each replicate block, totaling 75 flasks for each greenhouse. Seedlings were inoculated by inserting a single rice grain in each of three holes in the planting substrate that were positioned around the stem of the seedling and angled toward the rootball to a depth of 10 cm. A single flask of inoculum was used to inoculate

Table 2. Cultures utilized for virulence testing of *Phytophthora cambivora*, *P. cinnamomi*, *P. kelmania*, and *P. pini*. Isolate ID number corresponds to the number of cultures obtained for each of the four *Phytophthora* species. Location of origin indicates city and state where the isolate was recovered. Host of origin indicates the species of *Abies* from which each *Phytophthora* isolate was obtained.

<i>Phytophthora</i> species	Isolate ID	Location of origin	Host of origin	NCBI accession
<i>P. cambivora</i>	01	Stayton, OR	<i>A. procera</i>	KU053253
<i>P. cambivora</i>	02	Mason County, MI	<i>A. fraseri</i>	...
<i>P. cambivora</i>	03	Tacoma, WA	<i>A. procera</i>	KU053263
<i>P. cambivora</i>	04	Puyallup, WA	<i>A. procera</i>	KU053270
<i>P. cambivora</i>	05	Salem, OR	<i>A. procera</i>	KU220617
<i>P. cambivora</i>	06	Stayton, OR	<i>A. procera</i>	KU053251
<i>P. cambivora</i>	07	Maple Valley, WA	<i>A. procera</i>	KU053259
<i>P. cambivora</i>	08	Eatonville, WA	<i>A. procera</i>	KU053260
<i>P. cambivora</i>	09	Stayton, OR	<i>A. procera</i>	KU053252
<i>P. cambivora</i>	10	Enumclaw, WA	<i>A. procera</i>	KU053264
<i>P. cambivora</i>	11	Canby, OR	<i>A. procera</i>	KU053255
<i>P. cinnamomi</i>	01	Placerville, CA	<i>A. equi-trojani</i>	KU053228
<i>P. cinnamomi</i>	02	Placerville, CA	<i>A. concolor</i>	KU053229
<i>P. cinnamomi</i>	03	Placerville, CA	<i>A. nordmanniana</i>	KU053227
<i>P. cinnamomi</i>	04	Camino, CA	<i>A. nordmanniana</i>	KU053226
<i>P. cinnamomi</i>	05	Raleigh, NC	<i>A. fraseri</i>	...
<i>P. cinnamomi</i>	06	Raleigh, NC	<i>A. bornmuelleriana</i>	KU220613
<i>P. cinnamomi</i>	07	Raleigh, NC	<i>A. equi-trojani</i>	KU220612
<i>P. kelmania</i>	01	Litchfield, CT	<i>A. fraseri</i>	KU053233
<i>P. kelmania</i>	02	Groton, NY	<i>A. fraseri</i>	KU053242
<i>P. kelmania</i>	03	Groton, NY	<i>A. fraseri</i>	KU220615
<i>P. kelmania</i>	04	Groton, NY	<i>A. fraseri</i>	KU053244
<i>P. kelmania</i>	05	Manitowoc County, WI	<i>A. balsamea</i> var. <i>phanerolepis</i>	...
<i>P. kelmania</i>	06	Jefferson, NC	<i>A. bornmuelleriana</i>	KU053247
<i>P. kelmania</i>	07	Jefferson, NC	<i>A. fraseri</i>	KU053249
<i>P. pini</i>	01	Warren, CT	<i>A. balsamea</i> var. <i>phanerolepis</i>	KU053230
<i>P. pini</i>	02	Groton, NY	<i>A. fraseri</i>	KU053241
<i>P. pini</i>	03	Allegan County, MI	<i>A. fraseri</i>	AY995350
<i>P. pini</i>	04	Jefferson, NC	<i>A. fraseri</i>	KU053248

all seven fir species receiving a given treatment in a single block. After 8 weeks of incubation, a second inoculation was performed on living trees following the above protocol. This second inoculation was implemented as a precaution to ensure that surviving seedlings were not simply disease escapes resulting from the failure of inoculum to contact roots.

Incubation conditions. For the first 5 days immediately following both inoculation periods, overhead irrigation was applied at a rate of 0.25 ml/min for 60 min durations twice per day to create saturated soil conditions conducive to PRR (Kuan and Erwin 1980; Rhoades et al. 2003). After this initial saturation period, irrigation was reduced to a 20 min period, once daily. To comply with USDA Animal and Plant Health Inspection Service regulations, irrigation runoff was collected and disinfested with 8.25% NaClO. A 12-h photoperiod was maintained throughout the duration of the experiment using 400-W high-pressure sodium greenhouse lights at an average intensity of 178 $\mu\text{mol}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$. Greenhouse temperatures were programmed to remain within dictated degree ranges throughout a 24-h period with no predetermined diurnal fluctuations. Cooling was achieved through automatic activation of exhaust fans and evaporative cooling. To preempt the possibility of infestation by *Botrytis* spp., trees were treated once during shoot elongation with fludioxonil at a rate of 56.7 g (2 oz) per 378.5 liters (100 gal), and air circulation was maintained using four horizontal airflow fans positioned overhead in the four corners of each greenhouse. Ambient temperature and relative humidity were recorded with HOBO Pro data loggers (Onset Computer Corp., Bourne, MA) at the western and eastern ends of each greenhouse. Soil temperature was measured using HOBO 4-channel analog data loggers with probes inserted 7.6 cm into the soil of two individual seedlings in each block. Seedlings were undisturbed for 8 weeks following the first inoculation and then again for an additional 8 weeks following the second inoculation for a total

duration of 16 weeks for the experiment. This experiment was repeated once in the following year.

Assessments. The onset of budbreak for each species was noted, and foliar assessments were made biweekly beginning at the onset of symptoms in the most susceptible species (approximately 19 days postinoculation) to estimate PRR incidence. Foliar ratings were made on an ordinal scale of 1 = alive (green foliage, fully expanded new growth, full turgor), 2 = failing (wilt, chlorosis, failure to break bud, desiccation, partial necrosis), and 3 = dead (fully necrotic foliage or severely wilted past the point of likely recovery).

At the 16-week conclusion of the experiment, seedlings were destructively processed to assess disease severity on the roots. All seedlings were carefully removed from Deepots, and root systems were washed free of potting media, taking care to retain as much root tissue as possible in severely damaged seedlings. The percent of rot on the root system was visually assessed on a 1 to 4 scale where 1 = <25% rot, 2 = 25 to 50% rot, 3 = 50 to 75% rot, and 4 = >75% rot. To estimate relative biomass reductions in comparison with noninoculated treatments, root systems were detached from the stem at the collar of the highest root and dried at 70°C for 72 h prior to weighing. To verify *Phytophthora* as the causal agent of mortality, root tip isolations were made on a subset of seedlings from each treatment in each block.

Data analysis. Analyses of variance (ANOVAs) of the various data sets generated by this research and mean separations among treatments were performed with SAS 9.4 (SAS Institute, Cary, NC). Graphical representations of data were achieved using R Statistical Software (R Foundation, Vienna, Austria). Analyses of variance of lesion size data from virulence testing assays were performed to compare isolates within each of the four *Phytophthora* species individually, using PROC MIXED procedures with posthoc mean separations using Tukey's honestly significant difference. Data from the

Fig. 1. Mean lesion sizes produced on noble fir seedling stems 7 days post-inoculation with various isolates of four *Phytophthora* species. Isolate identities and states of origin are listed along the x-axis for each *Phytophthora* species. Letters above bars serve as a notation to indicate significantly different lesion sizes produced by the various isolates within a *Phytophthora* species, as determined by Tukey's honestly significant difference post-hoc analysis. A different type of letter notation was used for each *Phytophthora* species to indicate that isolates were not compared between *Phytophthora* species but only within a *Phytophthora* species. Information on isolates is provided in Table 2.

two trials were combined. For the greenhouse screening, data from the two greenhouses were assessed separately because of the lack of replication of temperature within a single year's trial. The three isolates of each *Phytophthora* species were also analyzed separately

in each analysis, owing to the selected method of physical randomization and spatial setup within each greenhouse. For all comparisons of response variables among hosts and treatments, data from the five-tree experimental unit for each fir species in each treatment within a

Table 3. Rho and *P* values from Pearson's product-moment correlation (PPMC) examine agreement of the three response variables assessed in this study (area under the disease progress curve [AUDPC], root rot rating [rot], and dry weight [wt.]) for each of the seven *Abies* species. Multiple linear regression (MLR) analysis indicates the predictive power of using rot and wt. to forecast AUDPC for each host species.

<i>Abies</i> species	Pearson's PPMC			MLR
	AUDPC versus rot	AUDPC versus wt.	rot versus wt.	AUDPC versus rot, wt.
<i>A. procera</i>	$r = 0.97$ $P < 0.0001$	$r = -0.74$ $P < 0.0001$	$r = -0.81$ $P < 0.0001$	$R^2 = 0.72$ $P(\text{rot}) = 0.0002$ $P(\text{wt.}) = 0.6326$
<i>A. fraseri</i>	$r = 0.95$ $P < 0.0001$	$r = -0.63$ $P = 0.0005$	$r = -0.79$ $P < 0.0001$	$R^2 = 0.79$ $P(\text{rot}) = 0.0004$ $P(\text{wt.}) = 0.0084$
<i>A. concolor</i>	$r = 0.86$ $P < 0.0001$	$r = -0.50$ $P = 0.009$	$r = -0.74$ $P < 0.0001$	$R^2 = 0.94$ $P(\text{rot}) < 0.0001$ $P(\text{wt.}) = 0.0014$
<i>A. balsamea</i>	$r = 0.86$ $P < 0.0001$	$r = -0.72$ $P < 0.0001$	$r = -0.80$ $P < 0.0001$	$R^2 = 0.95$ $P(\text{rot}) < 0.0001$ $P(\text{wt.}) = 0.039$
<i>A. balsamea</i> var. <i>phanerolepis</i>	$r = 0.86$ $P < 0.0001$	$r = -0.82$ $P < 0.0001$	$r = -0.74$ $P < 0.0001$	$R^2 = 0.92$ $P(\text{rot}) < 0.0001$ $P(\text{wt.}) = 0.0034$
<i>A. nordmanniana</i>	$r = 0.95$ $P < 0.0001$	$r = -0.83$ $P < 0.0001$	$r = -0.94$ $P < 0.0001$	$R^2 = 0.87$ $P(\text{rot}) < 0.0001$ $P(\text{wt.}) = 0.054$
<i>A. bornmuelleriana</i>	$r = 0.92$ $P < 0.0001$	$r = -0.80$ $P < 0.0001$	$r = -0.76$ $P < 0.0001$	$R^2 = 0.76$ $P(\text{rot}) < 0.0001$ $P(\text{wt.}) = 0.0608$

Fig. 2. Visual assessment of percent rot on the rootball caused by each *Phytophthora* species for each *Abies* species. The seven *Abies* species are arranged in vertical facets. *Phytophthora* isolates (1 to 3 for each species) are arranged in horizontal facets. The two temperatures are displayed adjacently but are not compared statistically. Lowercase letters above gray bars show mean separations between *Phytophthora* treatments at 27°C for the host indicated. Uppercase letters above black bars show mean separations between *Phytophthora* treatments at 15°C for the host indicated. Crosses on bars indicate treatments in which % root rot did not differ significantly from controls. In instances in which there were no statistical differences among the four *Phytophthora* species, letters are absent from above histogram bars. *Phytophthora* species: cinn = *P. cinnamomi*; kelm = *P. kelmiana*; and camb = *P. cambivora*.

block were averaged to constitute a single measurement. Because there were five blocks in each greenhouse, each fir × *Phytophthora* combination had a total of five replicates for each isolate and temperature.

Biweekly foliar assessments from the greenhouse screening were summarized as a time course of percent mortality within each host-treatment combination and used to construct disease progress curves (Madden et al. 2007). Mortality over time was used to calculate area under the disease progress curve (AUDPC), which in turn was used to compare mortality among host species in each treatment. AUDPC measurements were log transformed to achieve normality within a host species and linearize the data. The root rot severity categorical classes (1 to 4) were converted to their corresponding percentage ranges, and the midpoint value of each range was utilized for parametric statistical analysis (Campbell and Neher 1994; Horsfall and Barratt 1945). Dry weights, in grams, of the dried root systems were expressed as percentages of the control (noninoculated) treatments. ANOVA tests were performed to assess differences in each response variable among the different fir species within each treatment, as well as among the different treatments for each fir species individually. Pearson's product-moment correlation (PPMC) was used to assess harmony among the three response variables from the greenhouse *Abies* screening. For PPMC analysis, data from all trials, treatments, and temperatures were combined to understand how well the response variables represented each other, but they were also examined by each host species individually to assess differences. Root rot rating and dry weight response variables were regressed against AUDPC to test the null hypothesis that percent rot and root biomass remaining after infection are not indicative of disease development. This test was performed to gauge the predictive power of using data from these response variables to forecast disease progression.

Differences in radial growth rate of *Phytophthora* cultures at 15 and 27°C were compared using paired two-sample *t* tests with data from both trials combined. Unequal variances were assumed owing to differences between the two temperatures in the number of days required for a given culture to reach the perimeters of the Petri plate.

Average radial growth rates were also regressed against percent mortality of each tree species at each temperature to determine if the speed of pathogen growth was correlated to tree death.

Results

Selection of *Phytophthora* inocula from virulence testing. Lesions on noble fir stems produced by *P. cinnamomi* ranged from 87.9 to 124.3 mm among seven isolates. Eleven *P. cambivora* isolates produced lesions that ranged from 9.6 to 68.4 mm. Lesions ranged from 26.0 to 46.2 mm for seven *P. kelmiana* isolates and from 33.8 to 49.9 mm among four *P. pini* isolates. The three isolates of each *Phytophthora* species producing the longest lesions were selected, except for *P. cinnamomi*, for which one less-virulent isolate was included in further experiments (Fig. 1).

Evaluating root rot severity rating and dry weight measurement as useful metrics for describing disease. PPMC analysis indicated that the root rot severity and dry weight metrics were strongly correlated with both disease progress and with each other (Table 3). As disease progressed, root rot rating severity increased ($r = 0.93$, $n = 182$, $P < 0.0001$) and dry weight root biomass decreased ($r = -0.71$, $n = 182$, $P < 0.0001$). Similarly, as root rot severity increased, root biomass decreased ($r = -0.79$, $n = 182$, $P < 0.0001$). Overall significance of the multiple regression analysis ($P < 0.0001$) between the response and experimental variables dictated rejection of the null hypothesis; however, an insignificant *P* value for the dry weight variable indicated that only the root rot rating was a reliable predictor of disease progress (Table 3).

Quantifying root rot severity and mortality owing to inoculation with four different *Phytophthora* species. Observational comparison of root rot severity owing to *Phytophthora* treatment for each host (Fig. 2) suggested a trend of greater damage on host roots inflicted by *P. cinnamomi*, followed by *P. kelmiana*, *P. pini*, and *P. cambivora*. These trends were not absolute; for example, *P. pini* was quite virulent on noble and Fraser firs, causing nearly as much damage as *P. cinnamomi* and *P. kelmiana* in these more susceptible hosts. On the typically more resistant Turkish and Nordmann firs,

Fig. 3. Disease progress curves displaying mortality over 16 weeks for seven *Abies* species exposed to three isolates each of four *Phytophthora* species at two temperatures, 15 and 27°C. The x-axis indicates ratings (R1 to R8) made biweekly over the course of 16 weeks. Ratings were assessed by estimating the condition of the aboveground foliar parts of each plant on a 1 to 3 scale where 1 = alive, 2 = failing, and 3 = dead. The number of dead trees (ratings of 3) were tallied and averaged across the five trees constituting an experimental unit, as well as across the five replicate blocks, to obtain a total percent mortality for each treatment at each rating date.

root rot severity caused by *P. kelmiana*, *P. pini*, and *P. cambivora* was rarely different from what was observed on control seedlings (Fig. 2). *Phytophthora cambivora* was arguably the least damaging *Phytophthora*, differing significantly from the other three treatments in nearly every fir species except for noble fir. Disease progress curves representing mortality over time illustrate the speed with which even more resistant *Abies* reached measurable levels of mortality with *P. cinnamomi* treatments and the relatively innocuous behavior of *P. cambivora* on most species aside from noble and Fraser firs (Fig. 3).

Observing differences in root rot severity and mortality based on isolate choice. Although not comparable statistically, visual

Fig. 4. In vitro radial growth (mm/day) of three isolates each of four species of *Phytophthora* at the two temperatures employed in the greenhouse study. Student's t-tests were used to assess differences in growth of each isolate for each *Phytophthora* species between the two temperatures. Statistically significant differences in growth between the two temperatures for a given *Phytophthora* isolate are indicated by stars above the bars.

observation indicated that differences in root rot severity and mortality were minimal among distinct isolates of each *Phytophthora* species with the exception of *P. cinnamomi*. It was apparent that isolate 1 of this species caused less severe root damage and lower mortality rates than the other two isolates tested, particularly at cool temperatures and on more resistant hosts (Figs. 2 and 3).

Temperature effects on mortality, root rot, and pathogen vigor. Mortality was lower in the cooler greenhouse than under warmer ambient temperatures. Higher temperatures increased the speed and quantity of mortality of seedlings and increased the severity of root rot (Figs. 2 and 3). Replicated radial growth assays of all isolates of the *Phytophthora* species used in this experiment indicated that colony expansion on solid media tended to progress at a higher rate at 27 than 15°C, although the differences were not statistically significant in some isolates (Fig. 4). Regression of radial growth rate against percent seedling mortality at 15 and 27°C indicated moderate positive correlations (average $R^2 = 0.45$) for balsam, white, Canaan, and Fraser firs. Radial growth rate was only weakly correlated with mortality in Nordmann, Turkish, and Noble firs (average $R^2 = 0.22$).

Discussion

Virulence test inoculations on noble fir seedling stems showed that distinct isolates of a given *Phytophthora* species varied little in their ability to cause lesions under the experimental conditions. The exception to this trend was observed in *P. cinnamomi*, for which isolate 05 produced statistically smaller lesions on noble fir stems (Fig. 1). This isolate was nevertheless included in the greenhouse root-inoculation experiments as *P. cinnamomi* isolate 1 to parallel studies performed by collaborating researchers that utilize this culture in *A. fraseri* inoculation studies in North Carolina. Although the design of the greenhouse root-inoculation study did not arrange for statistical comparison between isolates, it was apparent that *P. cinnamomi* isolate 1 also produced less severe root rot ratings, slightly larger biomass measurements, and noticeably slower disease progress on hosts in the greenhouse screening. Parallels between *Phytophthora* virulence on host stems and aggressiveness on roots have previously been established (Weiland et al. 2010), and observed variation among isolates of a given *Phytophthora* suggests that virulence testing as a means to characterize pathogen isolates prior to experiment initiation may be an important foundation for *Phytophthora* inoculation studies.

Inoculations performed at warmer temperatures resulted in overall greater mortality, higher percentages of rotted roots, and larger reductions in dry weight than identical treatments at cooler temperatures. Observations of more rapid radial growth of *Phytophthora* cultures on solid media at warmer temperatures implied that the pathogen may have an increased ability to colonize root tissues at higher temperatures. This idea was tested statistically by regressing radial growth rate against percent seedling mortality for each tree species. Moderately strong positive correlations supported this concept for balsam, white, Canaan, and Fraser firs; however, weak R^2 values for Nordmann, Turkish, and noble firs indicated that radial fungal growth rate was not likely as instrumental in causing mortality in

Table 4. Average percent mortality of seven *Abies* species after 16 weeks of exposure to three isolates each of four *Phytophthora* species at two temperatures. Percentages represent totals from all five blocks for each treatment. Analyses of variance were performed using the PROC MIXED procedure with post-hoc analysis using Tukey's honestly significant difference to determine mean separations between fir species.^z

Abies species	15°C treatment												27°C treatment											
	P. cambivora			P. cinnamomi			P. kelmiana			P. pini			P. cambivora			P. cinnamomi			P. kelmiana			P. pini		
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
<i>A. procera</i>	28 a	44 a	60 a	16 a	72 ab	100 a	52 a	20 a	8 a	40 a	64 a	68 a	80 a	92 a	96 a	88 a	100 a	100 a	92 a	100 a	96 a	96 a	92 a	96 a
<i>A. fraseri</i>	20 b	24 a	32 b	8 a	76 a	80 b	40 ab	12 a	20 a	32 ab	76 a	68 a	32 b	64 b	56 b	88 a	100 a	100 a	100 a	96 a	92 a	88 ab	96 a	100 a
<i>A. concolor</i>	8 b	4 b	28 b	4 a	44 bcd	64 bc	12 ab	4 a	0 a	20 b	12 b	28 b	16 bc	12 c	28 c	12 c	76 c	88 b	56 b	52 b	40 b	64 b	60 b	64 b
<i>A. balsamea</i>	0 b	0 b	0 b	4 a	52 bc	40 cd	4 b	0 a	0 a	8 b	0 b	4 b	8 bc	8 c	0 c	48 b	92 ab	100 a	68 ab	84 a	32 b	28 c	32 bc	48 b
<i>A. balsamea</i> var. <i>phanerolepis</i>	0 b	0 b	0 b	0 a	12 cd	16 cd	0 b	0 a	0 a	8 b	0 b	0 b	0 c	0 c	0 c	44 bc	84 b	80 bc	36 bc	24 bc	16 bc	16 c	4 c	16 c
<i>A. nordmanniana</i>	0 b	0 b	0 b	0 a	8 d	28 cd	0 b	0 a	0 a	0 b	0 b	0 b	0 c	0 c	0 c	12 c	56 c	70 bc	0 c	0 c	0 c	0 c	0 c	0 c
<i>A. bornmuelleriana</i>	0 b	0 b	0 b	0 a	4 d	8 d	0 b	0 a	0 a	0 b	0 b	0 b	0 c	0 c	0 c	4 c	52 c	56 c	0 c	4 c	4 c	0 c	4 c	0 c

^z Letters next to measurements indicate significantly different mean mortality among the seven host species within each *Phytophthora* × isolate treatment.

these species. Similarly, when evaluated on a by-host basis, multiple linear regression indicated that root dry weight was inadequate for forecasting AUDPC in Nordmann, Turkish, and noble firs (Table 3). Because these results were observed in two of the most disease-resistant species (Nordmann and Turkish firs) and the most susceptible species (noble fir), it is speculated that disease dynamics during host-*Phytophthora* interactions for species that fall on the extremes of the resistance spectrum could be directed more by root phenology or molecular interactions rather than pathogen vigor or maintenance of root mass. The actual mechanism of disease resistance or susceptibility to *Phytophthora* in *Abies* is as yet unknown, but microscopic evaluation of *P. lateralis* Tucker & Milbrath infection of Port-Orford cedar (*Chamaecyparis lawsoniana* [A. Murray] Parl.) roots indicated that cortical cell invasion was retarded and vascular tissues were nonpenetrated in resistant roots, suggesting that resistance to PRR in conifers could be related to cellular interactions and root architecture (Oh and Hansen 2007). A concept that may explain the rendering of dry weight measurements inadequate for predicting host mortality includes that healthy roots may slowly regenerate over damaged roots, which would increase overall root biomass while still leaving the plant vulnerable to failure from disease. This occurrence has been observed in Douglas fir (*Pseudotsuga menziesii* [Mirb.] Franco) and Canaan fir (Frampton 1999; Hansen et al. 1980). Likewise, rapid mortality at warm temperatures could be owing to factors other than rapid fungal growth, such as increases in host transpiration rate owing to heat and a resulting deficit in moisture that is exacerbated by root deterioration.

Knowledge of how external factors affect symptom expression and potential resistance to PRR in the different species of *Abies* used as Christmas trees is critical to choosing the appropriate species to cultivate in high-incidence PRR sites. Findings from the greenhouse study indicate that Nordmann and Turkish firs are quite resistant to the four *Phytophthora* species tested but increase in sensitivity to *P. cinnamomi* as temperatures reach 27°C or greater. This trend implies that these species may do well in most parts of the country but are apt to fail in warm, moist areas where *P. cinnamomi* is pervasive such as the Piedmont region of North Carolina or irrigated Christmas tree fields in northeastern California. Canaan fir had root rot severity values statistically similar to resistant species but displayed greater disease progress than Nordmann or Turkish firs with *P. cinnamomi*, *P. kelmiana*, and *P. pini* at 27°C (Figs. 2 and 3, Table 4). These results suggest that Canaan fir may be an appropriate species to cultivate in high-risk PRR sites in cooler areas such as the Pacific Northwest and Great Lakes states. Experimental values measured for balsam and white firs were intermediate between susceptible and resistant species, depending on the *Phytophthora* species and temperature. At high temperatures and with virulent *Phytophthora* species such as *P. cinnamomi* and *P. kelmiana*, root rot severities for these *Abies* were high, whereas with *P. pini* and *P. cambivora*, particularly at lower temperatures, root rot severity corresponded more with resistant firs. Because *P. cambivora* and *P. pini* were the predominant *Phytophthora* species recovered from Christmas tree farms in the Pacific Northwest and New England, respectively (McKeever and Chastagner 2016), results from the greenhouse experiment suggest that cooler sites in these regions may be successful growing areas for these *Abies* species. Fraser and noble firs are recognized as the most PRR-susceptible species from the present study, suggesting that mortality may be expected with exposure to any of the *Phytophthora* species tested. However, if cultivation of these *Abies* hosts can be relegated to cooler sites, losses may be reduced to levels that may be acceptable in exchange for the high desirability of these species in the market.

The observed interactions among hosts, pathogen, and environment confirmed prior knowledge of the complexity of the PRR-*Abies* pathosystem and complemented previous research that indicates a lack of resistance in species such as Fraser and noble firs. Clarifications of how *Phytophthora* species and temperature affect host symptom expression may help to influence interpretation of ostensibly conflicting PRR research emerging from discrete geographic areas by acknowledging that the spectrum of resistances among species

of *Abies* should be considered individually for each unique region. Implementation of a multifactorial approach illuminated the importance of *Phytophthora* isolate virulence and documented the contribution of ambient temperature to disease development. Knowledge of how temperature influences physiology and pathogen function may assist nursery managers and growers to manipulate environment as a means of disease avoidance, and delineation of the conditions under which more moderately resistant *Abies* species are apt to thrive may increase their utility as Christmas trees in lieu of highly susceptible firs.

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