Resistance of half-sib Interior Douglas-fir families to *Armillaria ostoyae* in British Columbia following artificial inoculation.

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Abstract

The objectives of this study were: 1) to develop a methodology for screening conifer seedlings for resistance to *Armillaria ostoyae* (Romagn.) Herink, and 2) to screen a population of Interior Douglas-fir [*Pseudotsuga menziesii* var. *glauca* (Biessn.) Franco] population for resistance to *A. ostoyae*. Eighteen potted seedlings from each of 86 half-sib Interior Douglas-fir families were challenged with inoculum in a 3-year greenhouse trial. The seed originated from four geographically distinct tree breeding zones which represent physically and biologically different environments in southeastern British Columbia. Mortality and the final percent survival of inoculated trees showed differences among the families (survival range 0-61.1%) and breeding zones (survival range 6.6-25.3%). Maximum heritability index ($h^2 = 0.19$) occurred at 28 months. Survival analyses revealed that most of the differences in survival could be explained by the zone from which the family originated. The less susceptible seedlings originated from the drier and warmer zones and limited the spread of the fungus in the root system. Moderate levels of family variation in resistance to *A. ostoyae* and low-moderate heritability suggests that, in Interior Douglas-fir, gains in resistance can be achieved through breeding.

Introduction

There are currently more than 30 species of Armillaria occurring worldwide (Watling et al. 1991), many of which cause lethal levels of root disease in trees and agricultural crops. In general, losses attributed to Armillaria root disease are most severe in drier Mediterranean or continental climates (Kile et al. 1991). However, in the moister regions of the north temperate and boreal coniferous forests, one of these species, *Armillaria ostoyae* (Romagn.) Herink, causes lethal primary root disease and reductions in timber yield (Kile et al. 1991). In British Columbia (B.C.), tree mortality and growth losses that can be attributed to *A. ostoyae* are most severe throughout the moist southern interior. Variation in virulence did not explain the greater disease severity in the interior compared with coastal regions of B.C. (Morrison and Pellow 2002). Moreover, genetic differences between isolates from North America and Europe in the IGS-1 ribosomal region are small (White et al. 1998), which also suggests low variation in virulence. Alternatively, climate has been shown to be a major factor interacting with the relationship between hosts and *A. ostoyae* (Cruickshank et al. 1997; Morrison et al. 2000, 2001). While these studies provide fundamental information about virulence and climate relationships, little is known about the level of genetic variation in any of the hosts in response to any of the Armillaria species at the individual or population level.

In the B.C. Interior, Douglas-fir seedlings are deployed within defined breeding zones that serve as surrogates for the physical environment in which seedlings grow to their genetic potential. Planting outside of these breeding zones generally results in maladaptation (Ying and Yanchuk 2006). Therefore, environmental variation across these seed zones and host genetic variation within each zone may be factors that, in part, determine the type of Douglas-fir response to the fungus.

Over time, selection pressure from disease often increases the proportion of resistant trees within a population thereby affecting the extent, speed, or strength of defenses and the cost to the host; ultimately this can result in a difference in fitness along the range of susceptibility in individual plants (Franceschi et al. 2005). Conifer defense systems typically include: 1) constitutive responses consisting of resin accumulation, storage of toxins, and mechanical barriers, and 2) induced defenses involving chemical and structural changes in the host, both of which limit colonization (Franceschi et al. 2005). Tolerance, in contrast to resistance or susceptibility, is the ability of a plant to sustain pathogen damage without adversely affecting plant fitness. Plant resistance and tolerance have been proposed to be redundant strategies; however, both strategies rarely operate at high levels, but could be expressed together at lower levels (Simms and Triplett 1994; Fineblum and Rausher 1995). Changes in climate are expected to affect one or more of these host responses wherever the disease occurs.

In the present study we screened a seedling population of half-sibling Interior Douglas-fir families from four breeding zones for resistance to one isolate of *A. ostoyae*. The objectives of the study were to: 1) evaluate the efficacy of one inoculation technique, and 2) determine the level of genetic variation and heritability for resistance to *A. ostoyae* in a population of Interior Douglas-fir from the ICH zone of the southeastern B.C. By

achieving these objectives, we hoped to enhance our understanding of host population responses to Armillaria, and to provide information that may be adaptable to other related disease screening projects. This study represents an initial investigation into the variation of host response in Interior Douglas-fir.

Materials and Methods

Seedling material

Wind-pollinated, half-sib seed from the wild-stand parent tree seed inventory of the B.C. Interior Douglas-fir [*Pseudotsuga menziesii* var. *glauca* (Beissn.) Franco] tree improvement program was used in the study. The test population was comprised of parents with positive breeding values for growth. The majority of these high breeding value parents have been established in grafted clonal seed orchards for their respective breeding zone. A total of 86 families were randomly chosen from this test population to include 12 to 25 families from each of four Interior Douglas-fir tree breeding zones: Shuswap Adams (SA), West Kootenay low elevation (WKL) (< 1,000 m), West Kootenay high elevation (WKH) (>1,000 m), and Mica (M). These four tree breeding zones partition the Interior Cedar Hemlock biogeoclimatic zone (ICH) (Lloyd et al. 1990) mainly along clines of temperature and moisture, and are strongly associated with latitude. The SA and M zones occur in the north; the WKL and WKH in the south. In B.C. the ICH zone ranks second in terms of productivity after the Coastal Western Hemlock zone, and contains the richest diversity of tree species (Meidinger and Pojar 1991). The negative impacts of *A. ostoyae* are also most severe in the ICH (Lloyd et al. 1990).

Seedling preparation

In spring 1999, wind-pollinated seed from the 86 parents was germinated and grown for one season in 105-cc PSB415B Styroblock® containers at the B.C. Ministry of Forests and Range, Kalamalka Forestry Centre in Vernon, B.C. All seedlings were subsequently lifted and stored over winter at -2°C. In spring 2000, 25 seedlings from each family were transferred to 4.5-L plastic containers containing a mixture of 40 % sand, 30 % coarse forestry grade peat, and 30 % forest loam mineral soil from the grounds of the Forestry Centre in Vernon. After transplanting, a 3.75-cm.-diameter plastic tube was inserted into the soil mix such that the tube touched the bottom of the pot and continued above the top of the pot for approximately 2 cm. The seedlings were then grown outdoors in a lathe house while inoculum blocks of *A. ostoyae* were prepared. The layout of the lathe house consisted of 2500 pot positions on 22 benches. Each seedling was randomly assigned to a position in the retractable, plastic covered lathe house and remained in that position for the duration of the experiment.

Inoculum unit preparation and seedling inoculation

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Inoculum units were prepared at the Canadian Forest Service, Pacific Forestry Centre, Victoria, B.C. from freshly cut 225-g paper birch blocks (*Betula papyrifera* Marsh.) harvested from the B.C. interior. The technique was modified from the methodology outlined in Sturrock and Reynolds (1998). Initially, all 225-g blocks were autoclaved covered in warm water at 121^{o} C for 35 minutes and then put into autoclavable bags and autoclaved at the same temperature for 150 minutes. The blocks in the bags were set to cool in a sterile laminar flow hood overnight. The blocks were inoculated with an isolate of *Armillaria ostoyae* (Romagn.) Herink obtained from Morrison and Pellow (2002) by transferring a piece of the fungus from a 3 % malt extract broth agar plate (1.7 % agar) onto the bark at both ends of each block. Only one fungal isolate was used because of the number of blocks and the time needed to prepare and handle the blocks. The inoculated blocks were placed in plastic bags and stored in plastic storage boxes with a loose fitting lid for 2-3 years at 19^{o}C. After colonization, a 12–15-cm-diameter by 150-mm-long living oak branch segment (*Quercus garryana* Douglas) was inserted tightly into a hole drilled in one end of each block. Oak branches were prepared by removing lichens with a pressure washer and a scrubbing brush when needed, cutting to length, surface sterilizing using 10 % bleach for 10 minutes, and rinsing with tap water. The units were then placed in moist sand in plastic bins until the oak branch cambium became colonized with mycelium and rhizomorphs were formed (approximately 3-5 months). The units were then placed in moist vermiculite and transported to the Kalamalka Forestry Centre, Vernon, B.C.

In 2001 and 2002, a total of 1,536 seedlings from the 86 test families were inoculated with the inoculum units, with no family inoculated in more than one year (Table 1). The seedlings were inoculated in July and October 2001 and March and June in 2002. First, the plastic tube in each pot was removed and the inoculum branch segment was inserted into the hole with the attached birch block above the soil surface. The birch block was subsequently wrapped in a plastic bag to prevent desiccation. The soil mix was backfilled into the hole around the oak branch segment and lightly packed. The plastic tubes remained in the pots of all un-inoculated seedlings. Inoculated seedlings were exposed to the fungus for 3 years from the date of inoculation. Seedlings that were not inoculated remained in their lathe house position throughout the experiment. Temperature inside the lathe house was kept between 2 – 25^{o} C to provide optimum conditions for the fungus. In summer, the lathe house temperature was kept to a maximum of 25^{o} C by using regular water misting, fans, and by covering the lathe house with shade cloth. In winter, the lathe-house temperature was kept above 2 ^{o} C. Regular overhead irrigation kept the pots moist throughout the inoculation period. The seedlings were fertilized every 3 weeks following a standard Interior B.C. forest nursery regime. Periodic pruning of all trees kept the top of the seedlings below the 1-m-high irrigation booms.

Seedling measurements

Seedlings were inspected monthly for survival and the date of death was recorded. All seedlings that were inoculated and died were killed by *A. ostoyae*. Mycelial fans, which are indicative of *A. ostoyae*, were used to determine the infection status and to classify seedlings into three categories: uninfected, infected, or dead. At the end of 3 years, all living trees were transported to the Pacific Forestry Centre Laboratory in Victoria for destructive evaluation. These trees were removed from the pots and the soil was gently cleaned from their roots using water so that rhizomorphs and lesions from *A. ostoyae* could be identified. To more adequately describe disease damage, infected seedlings were further classified into two sub-categories: 1) girdled at the root collar and 2) un-girdled. Seedlings were considered dead when the foliage had turned bright red. The fungus was isolated from ten dead seedlings and tested for compatibility with the original isolate by pairing on 3% malt extract broth (1.7% agar). All isolates were vegetatively compatible (*i.e*. same genotype) with the original cultures as evidenced by the merging of mycelium of the two isolates (Guillaumin et al. 1991). The presence of callus at lesions was noted and aged by counting annual rings. At the soil line, the diameter (inside bark) of each living tree was measured using vernier calipers at two positions 90 degrees apart and the average of these was recorded for the stem diameter. Inoculum units were also surveyed for rhizomorph production and the presence of fungus in the oak stick and birch block.

Statistical analyses.

Statistical analyses were completed using the SAS statistical package (SAS Institute Inc., Cary, N.C. Ver. 9.1, 2002-2003). In all models, family was treated as a random effect, which was assumed to be independent and normally distributed. Within a given family, trees were assumed to respond independently of other trees in the same family. Models for binary responses (infection rates, etc.), which are described below, were fitted with Proc GLIMMIX (residual pseudo-likelihood), and seedling diameter, a continuous response, was analyzed with Proc MIXED (residual maximum likelihood estimation). McCulloch and Searle (2001) describe estimation for these types of models in more detail.

To determine if all families had a similar 3-year infection rate the following model was fitted:

$$[1] \quad \text{Prob}(Y_k = 1) = p = \frac{1}{1+\exp(a_0 + \beta_k)} \text{ and } \text{Prob}(Y_k = 0) = 1 - p$$

where Y_k is a binary (0, 1) variable denoting infection status after 3 years (not infected, infected) of a seedling selected at random from family k, a_0 is the fixed intercept, and β_k is the random family effect ($\beta_k \underset{i.i.d.}{\sim} N(0, \sigma_\beta^2)$). To test for host responses within breeding zone and inoculation year, model [1] was modified as follows:

$$[2] \quad \text{Prob}(Y_{ijk} = 1) = \frac{1}{1+\exp(a_0 + a_i + b_{j(i)} + \beta_{k(ij)})}$$

where Y_{ijk} is a binary response representing either survival, collar girdling, or callus presence for a seedling from inoculation year *i*, zone *j* and family *k*, a_0 is the fixed intercept, a_i is the fixed effect of inoculation year *i* (2001, 2002), $b_{j(i)}$ is the fixed effect of breeding zone *j* (M, SA, WKH, WKL) nested within inoculation year *i*, and $\beta_{k(ij)}$ is the random effect of family *k* nested within zone *j* and inoculation year *i*.

For analysis of the continuous diameter response, the breeding zone model had the following linear form:

[3] $$Y_{ijkl} = a_0 + a_i + b_{j(i)} + \beta_{k(ij)} + \varepsilon_{l(ijk)}$$

where Y_{ijkl} is the diameter of a seedling *l* in inoculation year *i*, zone *j*, and family *k*; a_0, a_i, $b_{j(i)}$, $\beta_{k(ij)}$ are analogous to the effects in Model 2, and $\varepsilon_{l(ijk)}$ is the random residual error represented by seedling *l* nested within family *k*, zone *j* and inoculation year *i* ($\varepsilon_{l(ijk)} \underset{i.i.d.}{\sim} N(0, \sigma^2)$).

Survival was analyzed by modeling the (discrete) hazard function h(*t*) (*t* =1, 2,…, 36 months), where h(*t*) = Prob(T = *t*|T > *t*-1) = probability a seedling dies in month *t* given that the seedling survives to the end of month *t*-1 and T = the total survival time (months after inoculation). The survival function can be obtained from the hazard function as follows:

S(*t*) = Probability of being alive at end of month *t* = Probability (T > *t*) = [1-h(1)] × [1-h(2)] × … × [1-h(*t*)].

A logistic hazard function with random family and fixed inoculation year, zone and season effects was fitted with Proc GLIMMIX (residual pseudo-likelihood):

[4] $$h(t_{ijkq}) = \frac{1}{1 + \exp\left[a_0 + a_i + b_{j(i)} + \beta_{k(ij)} + s_q + (c_0 + c_i + d_{j(i)})\log(t_{ijkq})\right]}$$

where: t_{ijkq} is the month since inoculation for a seedling from inoculation year *i*, zone *j*, and family *k; q* denotes the season into which the month falls; a_0 is the fixed intercept; a_i is the fixed effect of inoculation year *i*; $b_{j(i)}$ is the fixed effect of zone *j* nested within inoculation year *i*; $\beta_{k(ij)}$ is a random effect of family *k* nested within inoculation year *i* and zone *j*; s_q is a fixed effect allowing the hazard rate to shift depending on season (DJF,

MAM, JJA, SON) [*]; c_0 is a fixed parameter reflecting the overall influence of time (*i.e.* the logarithm of month since inoculation); c_i is a fixed effect allowing the time effect to vary among inoculation year; and $d_{j(i)}$ is a fixed effect allowing the effect of time to vary among zone *j* nested within inoculation year *i*.. Marginal (i.e., averaged over family effects) survival probabilities were estimated by 10,000 Monte Carlo simulations.

The proportion of living trees (survival) was estimated for each family for 36 monthly time periods by substituting the estimated parameters for fixed effects and the Best Linear Unbiased Predictors (BLUPs) of the random family effects into Equation 4 and calculating the corresponding survival function. A BLUP is an estimate of the random effect ($\beta_{k(ij)}$) for an individual subject (family *k* in this case) and describes how that subject differs from the population (zone) average. BLUPs were output from all models described above. Heritability for survival was estimated for each of the 36 time periods by computing the ratio *R/S* for zone and inoculation year, where *R* is the response, or gain, achieved in predicted survival by selecting the top 10 surviving families over the population mean survival, and *S* is the selection intensity value associated with the selection of the top 10 of 86 families (*S* = 1.7 from Table 2 in Becker 1984). Estimated log-odds ratios (Models 2 and 4) or mean differences (Model 3) comparing all pairs of zones were also calculated.

Results

1) Inoculation technique

Owing to inadequate colonization of the birch block, 55 (3.6%) of the inoculation units failed to be colonized by the fungus, meaning that 1481 seedlings were potentially exposed to the fungus (Table 1). Of these, 109 (7%) seedlings were not infected likely due to poor rhizomorph formation from the branch segment and 1,383 (93%) became infected (Table 2). In total, 334 trees were alive at the end of the 3 year test period, 109 of which were uninfected and 225 were infected (Table 2). After 3 years, no significant variation in infection rate was associated with family when all viable inoculum units were included in the data ($\sigma^2 = 0$, Eq. 1). The 109 inoculated but uninfected seedlings were removed from further analyses because they were not challenged by the fungal inoculum.

Fungal isolates used to culture the birch blocks were vegetatively compatible with cultures from 10 randomly selected dead seedlings. Two of the 55 un-inoculated seedlings died from unknown causes, and none of the 109 inoculated but uninfected seedlings died during the experiment. The un-inoculated seedlings were included to confirm the absence of Armillaria inoculum in the original soil mix. Since none of un-inoculated seedlings died or showed evidence of Armillaria infection, they were removed from the analysis. The 109 inoculated but uninfected seedlings were also removed from

[*] Acronyms DJF, MAM, JJA and SON represent months December January February, March April May, June July August, and September October November, respectively.

the analysis. Seedling mortality began 3 months after inoculation and the number of trees that died in any given month gradually increased, on average peaking at about 24 months after inoculum placement; however, the distribution of survival times (months) varied considerably among the four zones (Fig 1). The combined seasonal mortality rates for all three years were winter 9% (DJF), spring 28% (MAM), summer 37% (JJA), and fall 26% (SON). Root infection took place on roots 3 mm and greater and not at root hairs or wounds.

2) Family effects

The overall 3-year probability of survival after three years was 15.8% for the 86 families (range 0 – 56.3 %, Figure 2). At the end of the experiment, the top ranked family (8218) had 9 of 16 infected seedlings surviving, one of which was girdled but still alive and three contained callused lesions. The ten-worst ranked families all exhibited 100% mortality, while the ten top ranked families exhibited less than 65% mortality.

3) Breeding zone effects

At the end of 3 years, there were noticeable between-zone differences in survival, girdling, callusing at lesions, and the average time to death (Table 3). Seedling mortality occurred most rapidly in the families from the M and WKH zones, followed by the SA and WKL zones, respectively (Fig. 1). The majority of the seedlings from families originating in the SA and WKL zones survived until the second year after inoculum placement, while more than half the mortality in seedlings from the WKH and M zones had occurred by the second year (Fig. 1). Seedlings in families from the SA and WKL zones had higher survivorship classes and greater dispersion amongst the classes compared to families from the M and WKH zones (Fig. 2 and Table 4). Percent survival after 3 years did not differ significantly among breeding zones within inoculation year ($F= 0.46$, $p=0.63$, Eq. 2), but there were significant differences between the top two surviving zones (SA, WKL), which were inoculated in 2001, and bottom two surviving zones (M, WKH), which were inoculated in 2002 ($p<3.14 \times 10^{-5}$). Infected seedlings in families from the SA and WKL zones had the highest survival (22.8 and 25.3 percent, respectively), while seedlings in families from WKH and M zones had the lowest (7.7 and 6.6 percent, respectively Table 3). No significant variation among families within zones was detected ($\sigma^2 = 0.10 \pm 0.08$ SE, $p=0.19$, Eq. 2). Fewer families from the M zone were inoculated and those had a high mortality rate, so that only 13 inoculated seedlings from these families survived to the end of 3 years (Table 2).

At the end of 3 years, the percentage of seedlings that were girdled at the root collar did not differ among zones within or between inoculation years ($F=2.51$, $p=0.41$, Eq. 2) or among families within zones ($\sigma^2 = 0$). The percent callus did not different among zones within inoculation year ($F=0.52$ $p=0.60$ Eq. 2 Table 3), but seedlings from zone SA had significantly greater callus than from zone M and WKH ($p=0.08$ and $p=0.02$

respectively); no significant variation was found among families within zones ($\sigma^2 = 0.37 \pm 0.34$ SE, p=0.28, Eq. 2). Overall there were no significant differences in mean seedling diameter of the living seedlings between zones within inoculation year (F=1.39, p=0.23 Eq. 3), between seedlings from families within zones ($\sigma^2 = 0.29 \pm 0.34$ SE, p=0.19), or between zones across inoculation years (p>0.34).

4) **Survival analysis by breeding zone**

Survival analysis of the families within a breeding zone was performed using months after time since inoculum placement to clock time (Eq. 4). All fixed effects – inoculation year, zone within inoculation year, season, month, and the interactions between month and inoculation year, and between month and zone within inoculation year – were significant ($p<1.7 \times 10^{-4}$ Table 5). In any given month, inoculation year had the largest effect on survival followed by breeding zone (SA) within inoculation year (Table 6). The coefficient for the summer season (-0.19) confirmed the earlier results that summer had the lowest survival. The significant zone within inoculation year by time interactions confirmed that the shape of the survival curves were different among zones SA and WKL or M and WKH (Fig. 3). The family variance within zone ($\sigma^2 = 0.06 \pm .02$ SE, $\chi^2=14.0$, $p=1.83 \times 10^{-4}$) suggested that variation in seedling survival due to family within a breeding zone was significant, although this effect produces small changes in the predicted survival compared with those changes attributable to other fixed effects, as evidenced by the size of the family standard deviation (0.2) relative to the fixed effects coefficients. Family 8218 from zone SA had greater seedling survival compared to its zone mean survival (p=0.05) and family 8182 from zone SA and 9211 from zone M had lower seedling survival compared to their zone mean survival (p=0.05 and p=0.03 respectively).

To compare seedling survival rates between zones within and between inoculation years, differences in the log-odds of survival at month *t* compared with 2*t* (with all other fixed and random effects held fixed) were tested for all pairs of zones (Table 7). In this analysis, odds ratios greater than one (i.e., log-odds greater than 0) indicate an increase in the survival odds for one zone compared to the other, where odds in this case are the ratio of the predicted number of alive to dead seedlings at any time. Comparing between zones within and between inoculation year shows that the family survival curve is significantly different between all zones (p<0.05 Table 7). The odds of seedling survival varied the most between the WKL (inoculated in 2001) and the two wetter and colder zones (M and WKH, inoculated in 2002). The odds of seedling survival for zone WKL were approximately 3.8 to 5 times greater than for the M and WKH zones respectively and about twice that of zone SA (Table 7). The survival odds of seedlings from the SA zone were approximately twice the survival odds of seedlings from zone WKH, and 1.6 times greater than for seedlings from the M zone.

Our heritability index (HI) was generally low (< 0.05) for the first 12 months of the study; HI rose to a maximum of 0.19 at month 28, and subsequently declined rapidly

until month 36 (Fig. 4). Maximum HI was achieved at month 28 when overall survival had dropped to slightly less than 50 %.

Discussion

1) Inoculation technique

The inoculation units used in this study produced high infection rates and killed 84 % of the potted seedlings in 3 years. The paper birch inoculation blocks of the size used in this study appeared to be a suitable food base for the fungus, and most blocks remained firm after 2-3 years of colonization and 3 years of inoculation. Only one published study could be found that used birch as an inoculation unit for *A. ostoyae* (Piercey-Normore and Bérubé 2000) and results in that study were poor, possibly due to environmental factors during the field inoculation. In our study, live oak transfer sticks promoted rhizomorph formation probably because the stick was freshly cut, and the rough thick outer bark promoted good rhizomorph formation. A review of 12 Armillaria species inoculation studies[1] indicated that oak or sycamore gave the highest infection (55-97%), aspen was intermediate (43-54%) and alder was poor (11-28%). In the present study, tree mortality was the highest during the summer months, which is consistent with results from other studies (Omdal et al. 1995; Wilbur et al. 1971).

2) Survival by breeding zone and family

Armillaria species kill trees when the fungus spreads from its initial point of contact and girdles the root collar tissue (Garrett 1970). If the distribution of initial rhizomorph contact positions is similar across all breeding zones, then lesions that spread at the same rate should generate similar survivorship curves among trees of the same size. In this study, variation in both the initial contact positions and time of contact by the fungus should be minimal since the root systems filled and were bounded by the pots containing a homogenous soil mixture.

We had originally thought that we could inoculate all of the trees in the same year; however, this proved unattainable because of the time required for complete colonization in some of the transfer sticks. Seedlings from the two zones inoculated in the first year were later found to have the best survival in the study, which we believe was due to chance assignment rather than a real effect produced by the 2001 inoculation conditions. In our models, the effect labeled “inoculation year” actually comprises two potential sources of variation: changes in conditions that occurred between the two inoculation years (2001 and 2002) and, more importantly differences inherent to the two zones

[1] Davidson and Rishbeth 1988, Entry et al. 1991, Mallett and Hiratsuka 1988, Martin et al.1989, Morrison and Pellow 2002, Mugala et al. 1989, Omdal et al. 1995, Parks et al. 1993, Patton and Riker 1959, Piercey-Normore and Bérubé 2000, Redfern 1978, Rishbeth 1984.

inoculated in 2001 and those inoculated in 2002. Despite the potential confounding of inoculation year and zone effects, our results support the conclusion that there are important differences between all four zones. Differences between zones inoculated in the same year were statistically significant, and there were even larger differences between zones inoculated in different years. Although the latter cannot necessarily be attributed to zone effects alone, it seems unlikely that such comparatively large differences can be attributed solely to unknown factors associated with inoculation year. The seedlings were inoculated using identical procedures and were subsequently reared in closely controlled environmental conditions, spending 2 of the 3 study years together on the benches; therefore, although we can not measure the variation associated with inoculation year we expect it to be small.

Seedling diameter of the surviving trees was not different among families or zones indicating that tree size was probably not a factor in survival. In this study, no differences in seedling infection success were detected among families when all infected and uninfected trees were included, suggesting that the probability of infection was the same for all families. The removal of uninfected seedlings from the rest of the analyses is important because seedlings must be challenged by the pathogen in order for susceptibility to be determined.

We detected significant seedling survival differences among the half-sib families, but seedling survival was more broadly explained by sub-population or breeding zone effects. The major differences between the seedlings in the susceptible and less susceptible zones were the time at which initial mortality occurred and the rate of declining survival (Fig. 3). Seedlings from less susceptible zones (WKL and SA) appear to have some mechanism that slows lesion spread early in the infection process, as evidenced by the greater seedling survival after 3 years and longer time to the first seedling mortality. This lag in initial mortality may be important in developing an effective plant resistance response (Hudgins et al. 2005; Kuć 1982; Staskawcz et al. 1995). The seedlings from the more susceptible zones (M and WKH) appear to be slower in responding to infection, have a less effective response mechanism, or are not able to quickly recognize the fungus. Since the quantity of *A. ostoyae* inoculum, fungal genotype, and soil conditions were controlled for each tree, the differences in survival are likely due to genetic differences in seedlings from different breeding zones. One benefit of a defense mechanism that varies in speed of reaction is that the energy costs for individuals with lower susceptibility may be similar to those with greater susceptibility (Hoffland et al.1998); consequently, the benefits of defense would outweigh the costs.

Seedlings from the less susceptible zones had the highest survival rates after 3 years, but their rate of survival declined more rapidly than that of seedlings from the more susceptible zones in the last year. For small seedlings, slowing the fungal advance probably pushes the mortality curve into the future. However, for older and larger trees, the effect of slowing fungal advance may be very different. In larger root systems, lesions would occur further from the root collar, and this, combined with slower spread of the lesion and larger hosts, would require more time and energy by the fungus to kill the

tree. The likelihood of exhausting fungal inoculum before the fungus can girdle the roots or root collar would increase, resulting in some roots that would still be functioning and fewer girdled roots (less fungal inoculum). On older Douglas-fir roots, patch infections caused by *A. ostoyae* are common and typically contained to one side of larger roots, but the fungus can rapidly colonize and girdle smaller roots (Robinson and Morrison 2001). Slowing spread of the lesion relative to increasing root surface area would allow the infected tree to outgrow the fungus (Cruickshank et al. 1997; Hoffland et al. 1998; Jeger 1987). Such a strategy would provide increasing protection as the fungal attacks occur closer to the root collar because of the increasing root size. This is important because colonization of the root collar is a critical mortality factor since *A. ostoyae* can easily girdle a large number of roots from this position.

The frequency of callus on seedlings from the less susceptible zones (WKL and SA) was higher than on seedlings from the susceptible zones (WKH and M). Overall, the numbers of callused trees remained low probably due to the small size of the seedlings relative to the quantity of inoculum. Diminishing fungal reserves relative to the host reserves typically slows fungal spread (Garrett 1970) and this may allow sufficient time for host formation of callus tissues.

3) Environmental factors affecting host defense

The factors affecting host fitness caused by defense can be summarized as the probability of attack, the value of the tissue to the plant, and the benefit of defense (Hamilton et al. 2000, citing Mekey 1974). Compared to the wetter ICH subzones, the drier subzones receive as little as half the annual rainfall, about twice the growing degree-days >18^{o}C, and are warmer annually and in summer (Lloyd et al.1990). This pattern is consistent with zone effects derived from the overall survival analysis results among zones. A plant's root system plays a critical role in three functions: 1) the uptake and transport of moisture, nutrients, and metabolites, 2) structural support, and 3) storage (Kramer and Kozlowski 1979). Low soil moisture and high above-ground plant temperatures combine to reduce photosynthesis, leading to overall loss in carbon fixation and growth (Ericsson et al. 1996). Host functions would be affected by these conditions alone, but the effect would be exacerbated if portions of the root system are suddenly lost or damaged by a root pathogen (Desprez-Loustau et al. 2006).

Under the drier conditions that occur during part of the year in the SA and WKL zones, containing the fungus as soon as possible after infection would probably benefit host fitness because of the value of the roots. If host defense was linked to root tissue growth or *de novo* synthesis, then rapid detection and response in the early part of the year when moisture is not limiting would be important. Lesion surface area expansion is the highest under the environmental conditions that occur in the less susceptible SA and WKL zones (Cruickshank et al. 1997). Therefore, selection may favor the hosts that attempt to balance cost of disease with benefits of defense. Bliss (1946) points out that host resistance to *A. ostoyae* in a wide range of plant species is related to root growth that coincides with fungal activity, suggesting some link to an actively growing host. Douglas-fir root growth in the less susceptible breeding zones has been shown to begin sometime in May-June, followed by rapid root growth that becomes limited during

August and fall; on the other hand, fungal infections can occur at least until fall, and probably over the dormant period (Cruickshank et al. 2006). So slower root growth in summer months due to lower moisture might restrict effective host response to a shorter period prior to this. In the colder and wetter areas, the timing of fungal and tree growth may match more closely.

In the wetter and colder breeding zones (M and WKH), conditions may limit the speed and duration with which the fungus is able to expand the lesion front, which in turn could reduce the pressure for hosts to respond quickly. In wetter and colder areas, over most of the year the conditions for stump decay are probably below optimal. Higher stump moisture levels, especially those causing anoxia, and colder soil temperatures over much of the year could reduce fungal substrate use, inoculum longevity, and inoculum potential, thus reducing negative effects on host fitness. Inoculum potential is defined as the energy of growth of a parasite available for infection of a host at the site of the organ infected; in addition, the speed of infection is related to the quality of the inoculum (Garrett 1970). The fungus has difficulty surviving in soil conditions that are wet (Morrison et al. 2000, 2001) probably owing to the effects on the stump inoculum and lower probability of transfer at root contacts and poor rhizomorph growth (Cruickshank et al. 1997). Soil moisture extremes have a strong effect on Tomentosus root disease (Bernier and Lewis 1999), and flooding has been suggested as a control method for the root disease fungus *Phellinus noxious* (Chang 1996) and a range of wood inhabiting fungi that lack long-term survival structures (Chang 2003). High moisture and low temperatures (below 10^{o}C) inhibited growth of Armillaria species (Pearce and Malajczuk 1990) and other wood decay fungi (Griffith and Boddy 1991) possibly by altering the gaseous gradient near the fungi (Morrison 1976; Rishbeth 1978).

Alternatively, trees growing in areas where soil moisture is adequate over the growing season and where evapotranspiration is reduced may also tolerate some root loss to the disease without adverse fitness costs. Tolerance, if it operates under field conditions, might help explain the lower survival in the seedlings from wetter breeding zones in this study. Under natural conditions, there would be larger trees and colder and wetter soil environments possibly reducing fungal inoculum viability, inoculum potential and ultimately lesion spread. This may allow the host to cope with the losses of some root function.

4) Implications for forestry

This study indicates that genetic adaptations to infection by *A. ostoyae* in Interior Douglas-fir are structured largely between the warm and dry and the cool and wet biogeoclimatic zones, and that host adaptation plays a role mainly in the drier breeding zones. Although variation among families is low to moderate, it appears that genetic improvement for reduced susceptibility could be fruitful, particularly in the warm and dry zones. Also, if trials are assessed when mortality is about 50 % and heritability is the greatest (*i.e.* $h^2 = 0.19$ at 28 months), selection will become more efficient and testing costs will be reduced. In future, independent culling or combined index selection for

growth and resistance to *A. ostoyae* will be necessary to optimize genetic gains through tree breeding. Greater replication of individual families might also help to indentify increased numbers of families with significant departures from the population, and tests for tolerance should also be considered in future studies. We would like to point out that increased family replication is not likely to change the overall family variance; as a result, family would still likely remain a smaller effect than the zone effects.

Finally, climate change could be another critical concern for host resistance and ecosystem management. If temperature fluctuations affect soil moisture during the growing season (Rosenberg et al. 1989), or if rainfall patterns or snow pack are affected, especially in conjunction with temperature, this could alter host-pathogen relationships in each area. In the long run, this effect would be most noticeable in the wetter breeding zones since the frequency of Douglas-fir individuals that limit lesion spread is low in these areas. In the short run, this may increase the mortality among trees already infected in the drier zones until the fungal inoculum is also limited by climate. Given the considerable *A. ostoyae* inoculum distribution across Canada and the northern hemisphere and that climate probably limits damage in many areas, this could have consequences much larger than are currently anticipated.

Acknowledgements

We would like to thank Art Robinson of the Canadian Forest Service (CFS) and the Department of National Defense for access to oak tree branches used to make inoculum. We would also like to thank Duncan Morrison and Hugh Barkley (CFS), and Michelle Cleary (Southern Interior Forest Region, B.C. Ministry of Forests and Range) for discussions on aspects of the manuscript; Antoine Lalumière, Dominique Lejour, and Robert Paul (CFS) for measurements on the living seedlings; and Valerie Ashley (Research Branch, B.C. Ministry of Forests and Range) who established and maintained the test seedlings in the lathe house at the Kalamalka Forestry Centre.

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Table 1. Summary of Interior Douglas-fir seedlings from four tree breeding zones inoculated with *A. ostoyae*.

Zone[a]	Number of families	Number of seedlings inoculated by year			Number of failed inoculum units	Number of seedlings with viable inoculum
		2001	2002	Total		
M	12	0	215	215	7	208
SA	25	439	0	439	12	427
WKH	25	0	450	450	20	430
WKL	24	432	0	432	16	416
Total	86	871	665	1536	55	1481

[a] M = Mica, SA = Shuswap Adams, WKH = West Kootenay High, WKL = West Kootenay Low.

Table 2. Summary of Interior Douglas-fir seedling condition after a three year incubation period with *A. ostoyae*.

Zone[a]	Number of dead seedlings	Number of living seedlings			Number of seedlings with viable inoculum units		Seedlings with viable inoculum infected (%)
		Uninfected	Infected	Total	Uninfected	Infected	
M	182	13	13	26	11	197	94.7
SA	293	49	83	132	51	376	88.1
WKH	384	14	32	46	14	416	96.7
WKL	286	33	97	130	33	383	92.1
Total	1145	109	225	334	109	1383	92.7

[a] M = Mica, SA = Shuswap Adams, WKH = West Kootenay High, WKL = West Kootenay Low.

Table 3. Three-year summary of survival, callus formation, girdling and mean time to death of Interior Douglas-fir seedlings inoculated with *A. ostoyae*.

Zone[a]	Number of seedlings dead or girdled	Survival of inoculated seedlings (%)	Survival of infected seedlings (%)	Infected living seedlings forming callus tissue (%)	Infected living seedlings becoming girdled (%)	Mean time to death (months)
Mica	185	15.3	6.6	1.5	23.1	20.7
SA	329	33.3	22.8	4.8	30.1	25.5
WKH	397	14.7	7.7	1.4	40.6	20.5
WKL	329	33.8	25.3	3.2	44.3	28.6

[a] M = Mica, SA = Shuswap Adams, WKH = West Kootenay High, WKL = West Kootenay Low.

Table 4. Three-year range summary of actual inoculation success, infection and survival for Interior Douglas-fir families inoculated with *A. ostoyae*.

Zone[a]	Number of families	Range of seedlings per family (number)		Range of family survival by class (%)	
		inoculated	infected	inoculated	infected
Mica	12	16 – 18	14 – 18	0.0 – 25.0	0.0 – 14.3
SA	25	16 – 18	11 – 17	5.6 – 61.1	0.0 – 56.3
WKH	25	16 – 18	14 – 18	0.0 – 27.8	0.0 – 18.7
WKL	25	15 – 18	13 – 18	0.0 – 52.9	0.0 – 46.7

[a] M = Mica, SA = Shuswap Adams, WKH = West Kootenay High, WKL = West Kootenay Low.

Table 5. Fixed and random model effects (Type III) of the half-sibling family survival analysis for inoculation year, and inoculation year within breeding zones, month since inoculation (months), and season (MAM, JJA, SON, DJF). Family within zone was included as a random effect.

Effect	Numerator DF	Denominator DF	F Value	P
Inoculation year	1	3053	91.25	<0.0001
Zone within inoculation year	2	3053	9.53	<0.0001
Season	3	3053	48.45	<0.0001
Month	1	3053	712.24	<0.0001
Inoculation year x month	1	3053	64.73	<0.0001
Zone within inoculation year x month	2	3053	8.69	<0.0002
Family(zone)	1	-	14.00	<0.0001

Table 6. Parameter estimates for the survival analysis (Eq. 4) of half-sibling families within breeding zones over time since inoculation, season, and inoculation year. Family within zone and inoculation year was included as a random effect.

Effect			Estimate	Std. err.	DF	t	P
Intercept	a_0		7.10	0.32	3053	22.12	<.0001
Inoculation year	a_1	2001	8.51	0.92	3053	9.29	<.0001
	a_2	2002	0.00	.	.	.	.
Zone(inoculation year)	b_1	SA(2001)	-4.16	1.05	3053	-3.96	<.0001
	b_2	WL(2001)	0.00	.	.	.	.
	b_3	M(2002)	1.16	0.63	3053	1.84	0.07
	b_4	WH(2002)	0.00	.	.	.	.
Season	s_1	DJF	1.13	0.11	3053	9.87	<.0001
	s_2	MAM	0.07	0.08	3053	0.86	0.39
	s_3	JJA	-0.19	0.08	3053	-2.36	0.02
	s_4	SON	0.00	.	.	.	.
Month	c_0		-1.57	0.11	3053	-14.71	<.0001
Inoculation year × month	c_1	2001	-2.33	0.28	3053	-8.36	<.0001
	c_2	2002	1.17	0.32	3053	3.67	0.00
Zone(inoculation year) × month	d_1	SA(2001)	0.00	.	.	.	.
	d_2	WL(2001)	0.00	.	.	.	.
	d_3	M(2002)	-0.42	0.21	3053	-1.98	0.05
	d_4	WH(2002)	0.00	.	.	.	.
Family	σ^2		0.0629	0.0240			

[a] Zone acronyms: M = Mica, SA = Shuswap Adams, WKH = West Kootenay High, WKL = West Kootenay Low.; Season acronyms: DJF=December, January, February; MAM=March, April, May; JJA=June, July, August; SON=September, October, and November.

Table 7. Odds ratios for contrasts of half-sibling family survival rates between breeding zones that would occur over a doubling of the time since inoculation (t vs. 2t) for the model in Table 6.

Comparison [a]	t-value	Probability	Odds ratio
SA vs. M	2.85	0.0044	1.68
M vs. WKH	1.98	0.0475	1.34
WKL vs. M	6.07	<0.0001	3.77
SA vs. WKH	5.38	<0.0001	2.24
SA vs. WKL	-3.67	0.0002	0.44
WKL vs. WKH	8.36	<0.0001	5.04

[a] M = Mica, SA = Shuswap Adams, WKH = West Kootenay High, WKL = West Kootenay Low.

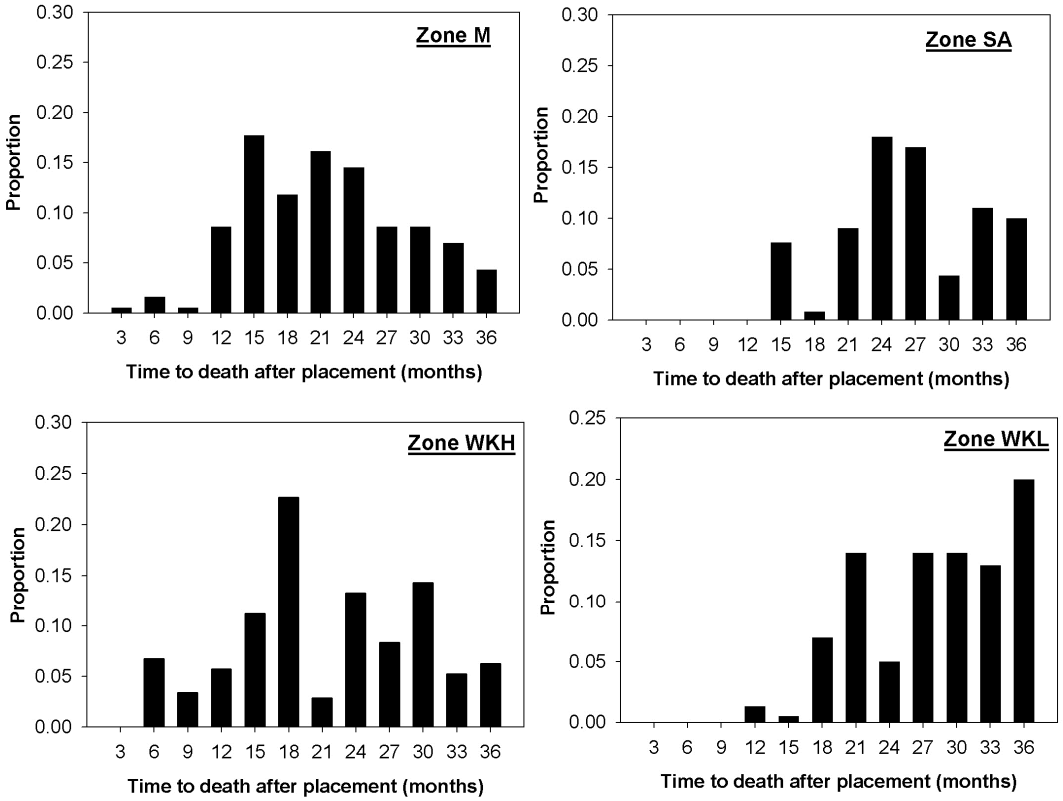

Figure 1. The proportion of seedlings that died every three months from the placement of the inoculum until the end of the 3 year experiment. Zone acronyms are defined in Table 1

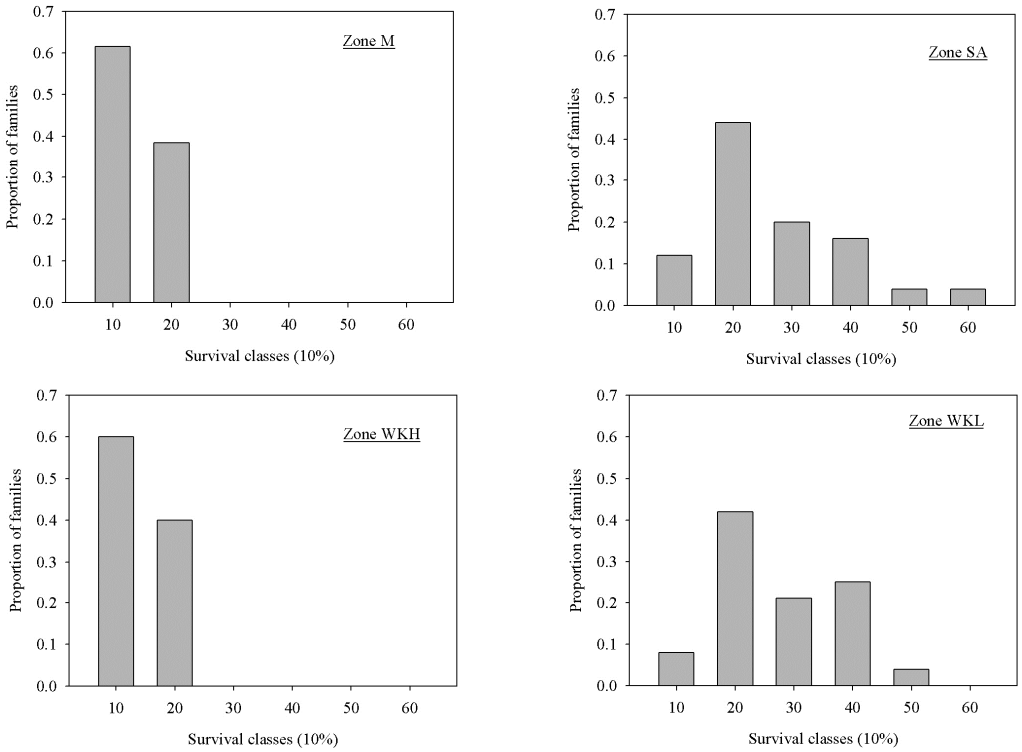

Figure 2. The proportion of families in survival categories (10% classes) for each breeding zone at the end of the 3 year experiment. Zone acronyms are defined in Table 1

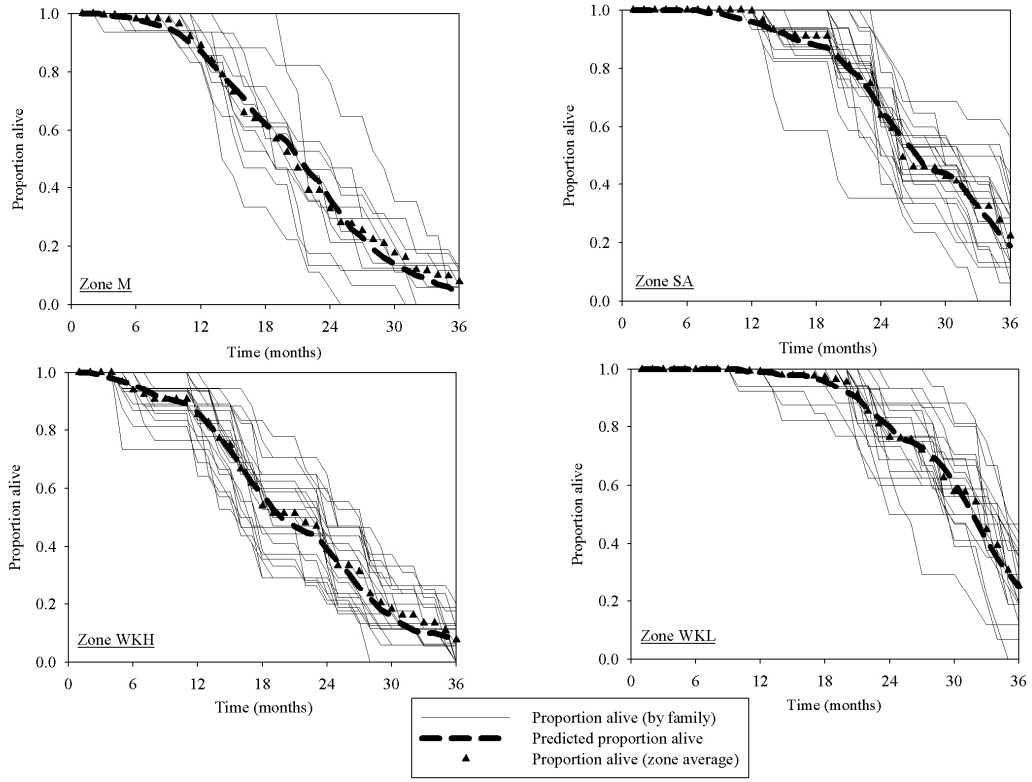

Figure 3. Family actual survival and predicted (Eq. 4) and actual zone average survival from the time of inoculum placement for each of four Interior Douglas-fir breeding zones.

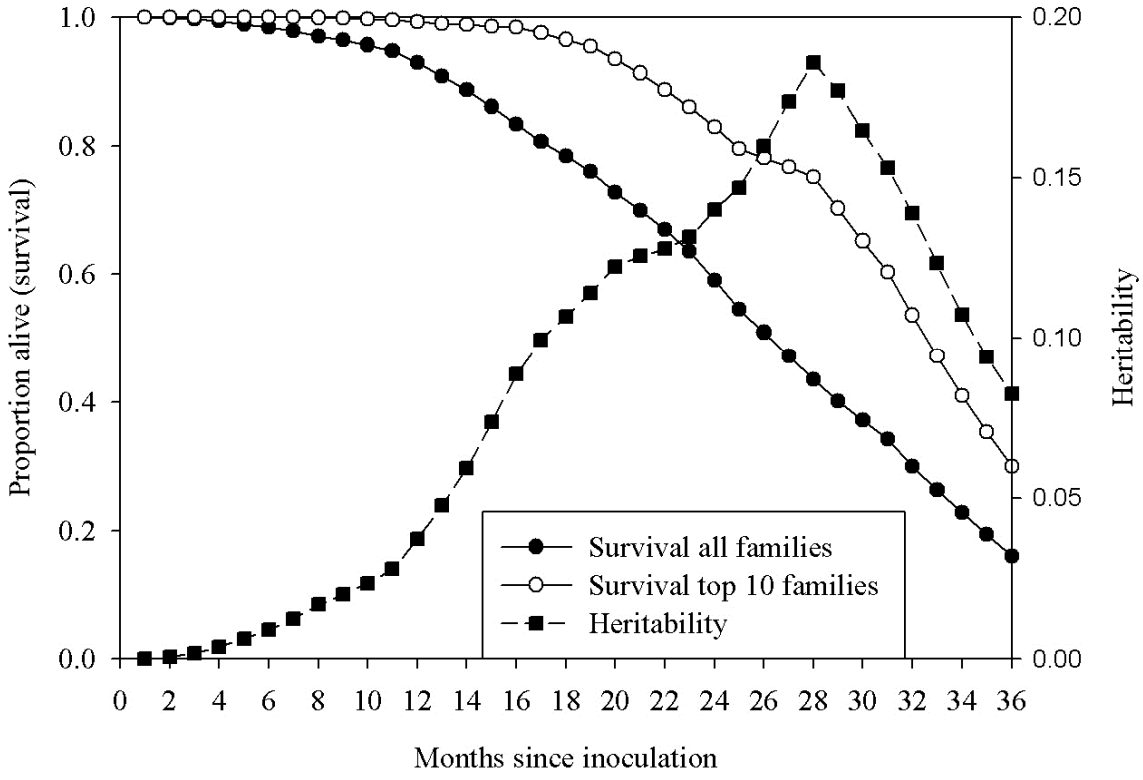

Figure 4. Predicted survival (proportion alive) for all 87 and the 10 top and bottom families, and heritability of survival of *A. ostoyae* inoculated Interior Douglas-fir seedlings at each of 36 months from the time of inoculation.