Response of Douglas-Fir to Infection by *Armillaria ostoyae* After Thinning or Thinning Plus Fertilization

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ABSTRACT

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Second-growth stands of Douglas-fir (*Pseudotsuga menziesii*) were thinned to a 5- × 5-m spacing (TT); additional plots were thinned and fertilized once with 360 kg of N (as urea) per hectare (TF). An unthinned, unfertilized stand (UT) served as a control. Ten years after treatment, trees were inoculated with two isolates of *Armillaria ostoyae*. Trees receiving the TF and TT treatments produced greater diameter growth, leaf area, and wood production per square meter of leaf area per year than did those under the UT treatment. Rates of infection by *A. ostoyae* were highest in trees that received the TF and lowest in trees that received the TT treatment. Concentrations of sugar, starch, and cellulose in root bark tissue were highest in trees receiving the TF treatment and lowest in trees receiving the TT treatment. Concentrations of lignin, phenolics, and protein-precipitable tannins were highest in root bark from TT trees and lowest in root bark from TF trees. Biochemical parameters of root bark tissue were regressed with incidence of infection; coefficients of determination (r^2) ranged from 0.07 (starch) to 0.57 (phenolic compounds). Ratios of the energetic costs of phenolic and of lignin degradation to the energy available from sugars (E_{pd}:E_{as} and E_{ld}:E_{as}) were correlated with incidence of infection (r^2 = 0.77 and 0.70, respectively). Thinning combined with fertilization may predispose *P. menziesii* trees to infection by *A. ostoyae* by lowering concentrations of defensive compounds in root bark and increasing the energy available to the fungus to degrade them.

Root disease caused by *Armillaria* spp. is a widespread and serious problem in forests of the United States (49). Many former pine stands have been converted to more disease-susceptible spruce and fir species as the result of past cutting practices and an 85-yr-old policy of fire suppression (3,18). Approximately 1% of commercial forest land in the northern Rocky Mountains is occupied by large active centers of root disease, and root disease causes mortality of at least three trees per hectare on another 13% (24). Trees of all ages and sizes are attacked, but the problem often is not recognized because early mortality is scattered and the trees are small. In the northern Rocky Mountains, most tree mortality from root pathogens is caused by *Armillaria* spp. (51), and infection by this fungus may be concentrated on certain sites and forest types (24,31).

Trees growing on nitrogen-limited sites may be particularly prone to attack by *Armillaria* spp. (43). Nitrogen deficiency or low light intensity may change the susceptibility of trees to pathogens, and multiple stresses may reduce a tree's defenses until it no longer can repel attack by *Armillaria* (38). Eight biological species of *Armillaria* have been identified in the northwestern United States and Canada (32), but *Armillaria ostoyae* (Romagn.) Herink seems to be one of the few *Armillaria* isolates infecting conifers (49).

Because *A. ostoyae* grows substantially better with sugars as a carbon source than on any other major substrate found in woody tissue (19), energy available from sugars in root bark tissue is the largest portion of energy available for growth. Lignin and phenolic compounds effectively inhibit growth of *A. ostoyae* (14). The inhibition of *A. ostoyae* by phenolic compounds depends on the concentration of the phenol and the concentration of sugars and nitrogen in the growing medium (14,47). Increased concentration of sugar and nitrogen may reduce or eliminate the inhibition of *Armillaria* growth by phenols (47). Energy required to degrade tree defense mechanisms, such as lignin and phenolic compounds, should contribute to the major portion of energy expended by the pathogen to colonize the tree root. Therefore, energy available to *A. ostoyae* from sugars (E_{as}) relative to energy required to degrade lignin (E_{ld}) or phenolic compounds (E_{pd}), expressed as a ratio (E_{as}:E_{ld} or E_{as}:E_{pd}), may be related to the frequency and severity of disease caused by *A. ostoyae*.

Our objectives in this study were twofold. First, we wanted to determine whether thinning or thinning combined with nitrogen fertilization would improve the physiological status of second-growth Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) and, thereby, increase resistance to infection by *A. ostoyae*. Second, we wanted to determine the role of tree defense mechanisms in resistance of *P. menziesii* to infection by *A. ostoyae*.

MATERIALS AND METHODS

Site descriptions. The Gold Hill site, in the Panhandle National Forest near Sandpoint, ID (lat. 48° 12′ N, long. 116° 30′ W), is at 954 m elevation, with a 35% slope toward the northeast. At the time of the study (September 1987 through September 1988), the site was dominated by 38-yr-old second-growth *P. menziesii*, 10.3 m tall on average. Excavation and inspection of the roots of 78 5- to 12-cm-diameter stumps of *P. menziesii* that remained after a 1977 thinning revealed no rhizomorphs or mycelial fans in the cambial tissue; the presence of such structures would indicate colonization by *Armillaria* spp.

The Tacoma Creek site, located on the Coville National Forest near Cusick, WA (lat. 49° 32′ N, long. 116° 43′ W), is at an elevation of 1,015 m, on a southwest exposure, with 15% slope. The site was dominated by 34-yr-old *P. menziesii*, 10–12 m high. Excavation and inspection of the roots of 73 3- to 11-cm-diameter stumps of *P. menziesii* that remained after thinning in 1977 revealed no rhizomorphs or mycelial fans in root cambial tissue.

The Solomon Mountain site, located on the Panhandle National Forest near Moyie Springs, ID (lat. 48° 48′ N, long. 116° 08′ W), is at 1,170 m elevation on a west exposure, with 10% slope. This site was dominated by 34-yr-old *P. menziesii*, with average height of 18 m. Excavation and inspection of the roots of 126 2- to 9-cm-diameter stumps of *P. menziesii* that remained after thinning in 1977 revealed no rhizomorphs or mycelial fans in root cambial tissue.

Sampling and analysis. The study was arranged in a randomized complete block design and replicated on three sites. Each tree was measured, sampled, and inoculated individually, providing a measure of variation among tree species within a site and among sites. Three treatments of second-growth *P. menziesii* were implemented in the fall of 1977 on 0.1-ha plots (42). Trees were thinned to a 5-m^2 spacing (TT), thinned to a 5-m^2 spacing, and fertilized at 364 kg of N (as urea) per hectare (TF) or left unthinned and unfertilized as control (UT). The fertilization study was completed in 1978. A strip of trees approximately 4-m wide, fertilized at the same rate, served as buffer near the plot borders. Plots were located at least 20 m apart to preclude fertilization affecting the thinned or control plots.

Ten years after fertilization, 10 trees on each of these plots were measured for height, age, diameter at breast height, diameter growth at breast height since fertilization and per year, sapwood basal area, and wood production per square meter of leaf area per year; sapwood basal area and wood production were determined as described by Waring (50).

Nutrient analysis. Ten trees in each plot were sampled in September 1987 for foliar nutrient content with methods described by Comerford and Leaf (10). Three composite samples of first-year needles were taken from the top, middle, and lower third of each tree. Three composite samples of root bark tissue from 0.5- to 3.0-cm-diameter roots were also taken. Samples were dried at 80 C and ground to <1 mm; a 1.0-g subsample of each was ashed at 525 C. The ash was dissolved in 6.0 ml of 1 N HCl, brought to 50 ml with deionized water, and analyzed for Al, B, Ca, Fe, Mg, Mn, K, P, S, and Zn (23) on a Jarrell-Ash 9000 inductively coupled plasma spectrometer (Jarrell-Ash, Waltham, MA). Total N was analyzed by standard micro-Kjeldahl techniques modified to include nitrate (5).

Biochemical measurements. Three trees on each plot, selected randomly from those sampled for nutrient analysis, were sampled in September 1987 for analysis of root bark. Roots 7–10 cm long and 0.5–3 cm in diameter were selected from each directional gradient of the tree (N 0–90°, E 90–180°, S 180–270°, and W 270–360°). The bark (living and nonliving periderm) was removed, dried at 80 C for 48 h, and ground to pass a mesh <1 mm. Samples (1.0 g) of ground bark tissue (one from each directional gradient) were pooled for each tree. A 0.2-g subsample was analyzed for total sugars and starch (22). Cellulose and lignin content were analyzed by procedures developed by Van Soest (44). Phenolic compounds were extracted from a 100-mg subsample with 10.0 ml of 80% aqueous acetone (v/v) for 24 h and analyzed by the method of Julkunen-Tiito (26), as modified by Kelsey and Harmon (27). Phenol standards were dissolved in 80% aqueous acetone (v/v). A 50-μl subsample of the 80% aqueous acetone extract (diluted 1:5 in 80% aqueous acetone) was analyzed for protein-precipitable tannins with a radial diffusion assay (21); the tannin standard was isolated from Douglas-fir bark (27).

Inoculum preparation. Three isolates of *A. ostoyae* were grown on 3% malt agar. Isolates JR 1953 and TY 186 were obtained from Jim Reaves of the Alabama Agricultural and Mechanical University, Normal (37). Isolate TY 186 was used only in determination of growth efficiency, as described below. Isolate DC1 was collected from a dying *Pinus monticola* Dougl. ex. D. Don. in the Deception Creek Experimental Forest in northern Idaho (16). These diploid isolates have been challenged against haploid isolates of known *Armillaria* spp. (2) and determined to be *A. ostoyae* (= North American Biological Species I). Blocks of red alder (*Alnus rubra* Bong., 2.0-cm-diameter × 10.0 cm long) were washed and placed in 10-L autoclave bags with 2 L of malt-extract medium. The blocks and medium were autoclaved for 60 min at 140 kPa, allowed to cool, and either isolate JR 1953 or isolate DC1 was transferred to the blocks. Blocks of *A. rubra* were incubated for 9 mo and were well colonized by the fungus before inoculation of trees.

Inoculation techniques. In the fall of 1987, 10 trees from each plot were inoculated with isolates JR 1953 and DC1. Each tree was inoculated four times, twice by a plug method and twice by a block method. The plug method involved drilling a hole (2.0 cm diameter × 10.0 cm deep) into a main root of each tree. A plug (1.75 cm diameter × 10.0 cm long) of *A. rubra* colonized by *A. ostoyae* was inserted into the hole. All plugs fit snugly and contacted the cambium and sapwood of each root. In the block method, roots 0.5–3.0 cm in diameter were inoculated by securely fastening a block (10.0 cm diameter × 20 cm long) colonized by *A. ostoyae* to the root. Four separate roots were inoculated on each tree. Each tree was inoculated with a block and a plug colonized with JR 1953 and a block and a plug colonized by DC1. Blocks and roots were wrapped in plastic to prevent desiccation of the inoculum block. Inoculated roots were covered with soil and left for 1 yr.

Verification of infection. After 1 yr, all inoculated sections of roots were removed and washed with tap water, followed by distilled water. Three sections approximately 2.5 mm long were surface-disinfested with 1% NaOCl solution for 7 min and then flamed for 5 s. Root sections were placed on 3% malt agar in 60-ml test tubes; tubes were capped and incubated in the dark for 9 wk at 20 C. All inoculum blocks and plugs were split in half, and recovery of *A. ostoyae* was attempted. Sections approximately 0.5 × 2.0 cm were immersed in 1% NaOCl for 7 min and then in 3% H_2O_2 for 5 min. *Armillaria* recovered from roots, plugs, and blocks was isolated and cultures were identified as *A. ostoyae* inoculated on the root, by diploid-diploid culture challenge with original cultures of DC1 and JR 1953 (1). Culture challenge was carried out on 3% malt agar in 100-mm-diameter petri dishes. Trees were rated for infection incidence based on the success of recovery of the fungus from each plug and block inoculation site: fungus not recovered from any inoculation site, 0; fungus recovered from one inoculation site, 1; fungus recovered from both inoculation sites, 2. Total incidence of disease was plug and block disease rate combined; thus, successful recovery from all four inoculation sites would be rated as 4.

Tests of growth of *A. ostoyae*. Samples of root bark (30 g) from three separate trees in each treatment on each site (27 total) were extracted with 900 ml of 80% aqueous acetone (v/v) for 24 h. The extract was evaporated with a rotary evaporator, brought to 300 ml with distilled deionized water, and sterilized by passage through a 0.2-μm Acrodisc filter (Gelman Sciences Inc., Ann Arbor, MI). Ten milliliters of this solution contained the phenolic equivalent of 1 g of root bark. Bark extract (50, 100, or 150 ml) was mixed with 10 g of glucose in sterile Melin-Norkrans (MNN) medium (30) and brought to 500 ml. This yielded media with 0.5:1.0, 1.0:1.0, and 1.5:1.0 g of root bark phenolic equivalents per gram of glucose. Each mixture was adjusted to pH 5.6 with 1.0 N HCl or 1.0 N NaOH, poured into 100-mm-diameter plastic petri dishes, and left to cool. One plug (3.0 mm diameter) from the margin of stock cultures of each of the isolates of *A. ostoyae* was transferred to each petri dish. Plugs contained no visible rhizomorphs. Cultures were incubated in the dark at 23 C, and the diameter of hyphal growth was measured at 3, 6, and 9 wk. Fungal biomass after 9 wk of growth was obtained by transferring cultures to glass beakers, adding 100 ml of deionized water, and steaming at 210 C to liquefy the agar (8,37). Colonies then were removed and rinsed with boiling water, and the water was removed by filtration through a 0.2-μm glass-fiber filter. The colonies were oven-dried at 80 C for 48 h and weighed.

Growth of *A. ostoyae* on root bark tissue. A factorial experiment was arranged in a randomized complete block design, with three treatments × three sites (blocks) × root bark from three trees (replicates) × three fungal isolates × five observations. The experiment was replicated three times.

A 1.0-g subsample of the ground root bark collected from each treatment at each site was placed in a 120-ml prescription bottle, brought to 100% moisture content with distilled deionized water, and autoclaved for 30 min. After cooling, *A. ostoyae* was transferred to the prescription bottles. Inoculum was cut from stock subcultures with a sterile cork borer (3.0 mm diameter); plugs contained no visible rhizomorphs. Bottles were capped with sterile, serum bottle caps and incubated at 23 C in the dark. Evolved carbon dioxide was measured with a Hewlett-Packard 5730 gas chromatograph (Hewlett-Packard Inc., Avondale, PA) 3, 6, and 9 wk after the experiment was initiated. Five prescription bottles in each treatment, prepared as above but without fungus, were used as reference blanks for all growth measurements. In addition, the fungus was grown in 60 prescription bottles, each containing 30 ml of 3% malt agar without root bark. Carbon dioxide evolution and fungal weight from this group were measured at 9 wk.

Growth efficiency of *A. ostoyae*. The growth efficiency of isolates JR 1953, TY 186, and DC1 of *A. ostoyae* was calculated by growing each isolate in sugar concentrations of 10, 20, or 40 g L^{-1} in MNN medium (30). The carbon source (glucose, fructose, or sucrose) was sterilized separately by dry autoclaving and added to the basal medium after the latter had cooled to 45 C (19). Plugs (3.0 mm diameter) cut from stock cultures on 3% malt agar were transferred to 30 ml of medium in 180-ml medicine bottles capped with 20-mm serum bottle stoppers. Disks contained no visible rhizomorphs. Cultures were incubated for 9 wk at 23 C in the dark. Mycelial dry weight was determined as described above. The equation used to determine growth efficiency (9) was:

$$\text{Growth efficiency} = \text{Mycelial dry weight/carbohydrate consumed} \times 100.$$

The experiment was arranged in a completely randomized factorial design; carbon source, carbon concentration, and isolates of *A. ostoyae* were treatments. The experiment contained five replicates and was conducted four times to ensure reliability of the results. Sugar concentration in the medium at the end of the experiment was determined by diluting 1 ml of medium with 100 ml of deionized distilled water and analyzing it as described by Hansen and Moller (22). Spectrophotometry was done on a Beckman DU-40 spectrophotometer (Beckman Instruments Inc., Irvine, CA) at 625 nm.

Calculation of thermochemical budget. The enthalpy of formation (ΔH_f) of any compound is the energy of reaction by which it is formed. The energy required to break a chemical bond between two compounds can be calculated by subtracting the ΔH_f of the first compound from the ΔH_f of the second compound. All ΔH_f measurements are expressed in kilojoules per mole (kJ mol^{-1}).

To obtain an accurate balance between energy obtained from sugars and the total work required for the fungus to degrade phenolics and lignin and colonize the tree, we calculated the ratio of energy required to degrade phenols (E_{pd}) or lignin (E_{ld}) to the energy gained from available sugars (E_{as}) in the root bark of host trees. The formula for E_{as} is:

$$E_{as} = [\Delta H_{f_{\text{glucose (mol)}}} + 6\Delta H_{f_{O_2\text{ (mol)}}} - (6\Delta H_{f_{H_2O\text{ (mol)}}} + 6\,\Delta H_{f_{CO_2\text{ (mol)}}})] \times \text{average growth efficiency of } A.\ ostoyae \text{ isolates} \times \text{mol sugar g}^{-1} \text{ root bark},$$

$$E_{as} = [-1273.30 + 6(-498.4) - (6(-241.8) + 6(-391.51))] \times 0.137 \times \text{mol sugar g}^{-1} \text{ root bark},$$

$$E_{as} = -63.54 \text{ kJ mol}^{-1} \times \text{mol sugar g}^{-1} \text{ root bark}.$$

The growth efficiency of the isolates (0.137) was averaged for the types and concentrations of media described in the preceding section. The concentration of sugars in root bark tissue was measured as described under biochemical measurements.

Energy of dissociation of phenolic compounds (E_{pd}) was calculated from the energy required for a molecule of an aromatic compound to be degraded through the ortho pathway for fungi via catechol (7,29). Values of ΔH_f stable organic compounds are found in Pedley et al (36). Enthalpy of formation of catechol is found in Khadikar et al (28) and of muconate in Dolbneva et al (13).

$$E_{pd} = \Delta H_{f_{\text{catechol}}} - \Delta H_{f_{\text{oxoadipate}}} - \Delta H_{f_{\text{succinate}}} - \Delta H_{f_{CO_2}} \times \text{mol phenol g}^{-1} \text{ root bark},$$

$$E_{pd} = -(374.11) - (-917.20) - (-1026.20) - (-469.8) - (-391.51) \times \text{mol phenol g}^{-1} \text{ root bark},$$

$$E_{pd} = 2430.60 \text{ kJ mol}^{-1} \times \text{mol phenol g}^{-1} \text{ root bark}.$$

The concentration of phenolic units in root bark tissue was measured as described under biochemical measurements.

Energy required to degrade lignin (E_{ld}) was calculated from the energy required to degrade a molecule of tyrosine through the ortho pathway for fungi via catechol (7,11). Enthalpies of formation (ΔH_f) of stable organic compounds are derived from Pedley et al (36), Dolbneva et al (13), and Khadikar et al (28), as stated above.

$$E_{ld} = \Delta H_{f_{\text{tyrosine}}} - \Delta H_{f_{\text{phenol}}} - \Delta H_{f_{\text{catechol}}} - H_{f_{\text{muconate}}} - \Delta H_{f_{\text{oxoadipate}}} - \Delta H_{f_{\text{succinate}}} - \Delta H_{f_{CO_2}} \times (\%\text{ lignin g}^{-1} \text{ root bark} \times \%\text{ tyrosine (w/w) in lignin}),$$

$$E_{ld} = (-695.10) - (-165.10) - (-374.11) - (917.20) - (-1026.20)\,(-469.80) - (-391.50) \times (\%\text{ lignin g}^{-1} \text{ root bark} \times \%\text{ tyrosine [w/w] in lignin}),$$

$$E_{ld} = 2658.82 \text{ kJ mol}^{-1} \times (\%\text{ lignin g}^{-1} \text{ root bark} \times \%\text{ tyrosine [w/w] in lignin}).$$

TABLE 1. Growth of second-growth *Pseudotsuga menziesii* 10 yr after treatment[a]

Treatment	Height (m)	dbh[b] (cm)	GSF[c] (cm)	SBA[d] (cm^2)	Vigor (wood produced m^{-2} leaf area yr^{-1})	LAI ($m^2\ m^{-2}$)[e]	Sapwood/heartwood ratio ($cm^2 cm^{-2}$)[f]
Thinned (TT)	15.3 a	8.2 b	3.05 b	186 b	93 b	6.3 b	1.9 b
Thinned and fertilized (TF)	15.8 a	9.2 b	3.94 c	232 c	102 b	7.9 c	2.9 c
Unthinned control (UT)	13.6 a	5.5 a	1.10 a	66 a	49 a	2.2 a	1.1 a

[a] Values are means of 30 samples. Within each column, values followed by the same letter are not significantly different ($P \leq 0.05$), as determined by Tukey's honest significant difference test.
[b] Stem diameter measured at breast height.
[c] Radial growth of stem since fertilization, measured at breast height.
[d] Sapwood basal area, measured at breast height.
[e] Leaf area index: Foliage area of the upper needle surfaces projected downward to a unit area of ground beneath the tree; measured in square meter of leaf area per square meter of ground.
[f] Area of sapwood relative to area of heartwood, calculated from two increment cores taken at N and E quadrants, 1.4 m aboveground.

The concentration of lignin in root bark tissue was measured as described under biochemical analysis. The ratios then were computed as E_{pd}:E_{as} and E_{ld}:E_{as}.

Statistical analysis. Normal distribution of the data was confirmed by univariate procedures. Disease percentages required a natural log transformation to normalize the data. The data were analyzed by ANOVA for a randomized complete block design (41). Residuals were normally distributed with constant variance. Individual treatment means were computed with Tukey's honest significant difference (HSD) test at $P \leq 0.05$.

RESULTS

Growth measurements. Tree height did not differ significantly among the three treatments (Table 1). Diameter at breast height, sapwood basal area, and vigor were significantly greater in trees from TF and TT stands than in trees from control stands (UT). Stem growth since fertilization, leaf area, and sapwood/heartwood ratio were significantly greater in trees from TF stands than in trees from TT stands or the UT control. These same three parameters were greater in TT trees than in the control.

Nutrients. Foliar concentrations of total N, P, K, Ca, Mg, and S did not differ for trees in any treatment (Table 2). Concentrations of Mn, Fe, and B were lower in foliage of trees from TF stands than in the UT control. Foliar concentrations of Mn did not differ between TF and TT stands, or between TT and UT stands. Concentrations of B were lowest in the foliage of TF trees and greatest in the UT controls. Foliar concentrations of Zn were highest in TT trees, whereas the lowest amounts were observed in TF trees. Roots of trees in TT stands had lower concentrations of total N than roots of trees in TF stands or the UT control.

Physiological responses. Concentrations of sugar and starch were higher in the root bark of TF trees than in TT trees. However, concentrations of sugars and starch in root bark did not differ in TT and UT trees (Table 3). Cellulose concentration was higher in root tissue of TF and UT trees than in TT trees. Concentrations of lignin, phenolic compounds, and protein-precipitable tannins were highest in root bark tissue of TT trees, as was the phenolic/sugar ratio.

Infection. In many, but not all, infected root segments, strands of white hyphae 0.5–1.0 cm long emerged from the point of contact with the inoculum block and advanced in the cambial zone of the root parallel to the axis; hyphae were only visible with the aid of a dissecting microscope. Small areas of resinosis (0.2–3.0 cm) surrounded the point of contact. Isolates of *A. ostoyae* recovered from roots were the same isolates used to inoculate the blocks of *A. rubra*, as shown by diploid-diploid crossings on 3% malt agar (1).

Hyphae of *A. ostoyae* sometimes, but not always, grew into the cambial zones of roots of *P. menziesii* when the plug inoculation method was used. Hyphae of *A. ostoyae* grew more often in the sapwood area of the roots.

Incidence of infection. Because disease rates and infection percentages between the two isolates of *A. ostoyae* and among the three sites were not significantly different, results are presented with combined data for isolates and sites.

Thinned and fertilized stands of *P. menziesii* appeared to be more susceptible to infection by *A. ostoyae*. Disease rates after block and plug inoculations were highest in TF stands. Root bark tissue and phenolic extracts from TF stands also stimulated the most hyphal growth and CO_2 evolution in cultures of *A. ostoyae*.

Block, plug, and total incidence of infection were significantly higher in TF trees than in TT or UT trees (Table 4). Plug and total incidence of infection were significantly higher in UT trees than in TT trees; block incidence of infection did not differ between these treatments. Total incidence of infection was not correlated with sugar, starch, cellulose, total extractable phenolics, protein-precipitable tannins, or the sugar/phenolic ratio in root bark tissue in either a linear or curvilinear relationship (Table 5). Lignin concentration was correlated with plug ($r^2 = 0.53$) and total ($r^2 = 0.57$) incidence of infection in both inverse linear and curvilinear relationships. Sugar concentration was correlated with block incidence of infection in positive linear ($r^2 = 0.47$) and curvilinear ($r^2 = 0.63$) relationships (Table 5).

The E_{pd}:E_{as} ratio was correlated ($r^2 = 0.77$) with the total incidence of infection in a curvilinear relationship (Fig. 1). The E_{lg}:E_{as} ratio also was correlated with total incidence of infection ($r^2 = 0.70$) in a curvilinear relationship (Fig. 2).

Growth of *A. ostoyae* on medium amended with root bark extract. Analysis of variance indicated that the second-order interactions of hyphal growth × fungal weight × isolate were not significant, whether isolates were grown on basal medium or on

TABLE 2. Elemental composition of needles of *Pseudotsuga menziesii* in 1987, 10 yr after silvicultural treatment and before inoculation with *Armillaria ostoyae*[a]

Treatments	Total N		Elements ($\mu g\ g^{-1}$ needle biomass)								
	Foliar	Root	P	K	Ca	Mg	Mn	Fe	B	S	Zn
Thinning (TT)	10,267 a	7,701 a	1,634 a	5,450 a	8,501 a	1,165 a	285 b	89 a	23.30 b	948 a	23.21 b
Thinning and fertilization (TF)	10,000 a	4,022 b	1,632 a	5,540 a	8,209 a	1,226 a	256 a	90 a	18.87 a	892 a	17.87 a
Unthinned control (UT)	10,399 a	7,344 a	1,621 a	5,459 a	8,876 a	1,157 a	297 b	110 b	25.89 c	879 a	20.10 b

[a] Values are means of 90 samples (three composite foliage samples per tree × three sites). Within each column, values followed by the same letter are not significantly different ($P \leq 0.05$), as determined by Tukey's honest significant difference test.

TABLE 3. Biochemical measurements of bark collected in 1987 from roots (0.5–3.0 cm in diameter) of second-growth *Pseudotsuga menziesii*, 10 yr after silvicultural treatment and before inoculation with *Armillaria ostoyae*[a]

Treatment	Sugar ($mg\ g^{-1}$ tissue)	Starch ($mg\ g^{-1}$ tissue)	Cellulose (%)	Lignin (%)	Phenolics[b]	Protein-precipitable tannins[c]	Phenolic/sugar ratio[d]
Thinning (TT)	23.61 a	34.52 a	35 b	60 b	254 b	164 b	17.19 b
Thinning and fertilization (TF)	40.97 b	45.26 b	51 a	40 a	203 a	127 a	5.73 a
Unthinned control (UT)	25.47 a	37.92 ab	44 a	47 a	144 a	104 a	10.32 ab

[a] Values are means of 27 samples (three root samples × three trees × three sites). Within each column, values followed by the same letter are not significantly different at $P \leq 0.05$, as determined by Tukey's honest significant difference test.
[b] Calculated as phenol equivalents per gram root bark tissue.
[c] Expressed in milligrams of I tannin per gram of root bark tissue.
[d] Calculated as phenol equivalents in root bark/milligrams of sugar extracted from root bark.

medium amended with root bark extract. Therefore, we will discuss only response to extract concentration and silvicultural treatment.

Hyphal growth and mycelial dry weight of *A. ostoyae* were highest when root bark phenolics and glucose were added to the basal medium at a ratio of 0.5:1.0 (Table 6). At root bark phenolic equivalent/sugar ratios of 1.0:1.0 and 1.5:1.0, hyphal growth and mycelial dry weight were greater after growth on medium amended with root bark phenolics from TF trees than after growth on medium amended with phenolics from UT or TT trees. Hyphal growth and mycelial dry weight were greater after growth on medium amended with root bark phenolics from UT trees than from TT trees. At a root bark phenolic equivalent/glucose ratio of 1.5:1.0, hyphal growth and mycelial dry weight were inhibited regardless of silvicultural treatment; root bark phenolics from TT trees inhibited hyphal growth and dry weight of *A. ostoyae* most effectively.

Growth of *A. ostoyae* on root bark. Three weeks after inoculation, CO_2 evolution did not differ significantly in *A. ostoyae* growing on ground root bark from trees in stands that received any silvicultural treatment (Table 7). After 6 and 9 wk, evolution of CO_2 was greatest from isolates of *A. ostoyae* growing on root bark from TF trees and least from isolates growing on root bark from TT trees. Production of carbon dioxide was correlated positively with weight of *A. ostoyae* growing on 3% malt agar alone ($r^2 = 0.84$, $n = 30$):

$$\text{Weight} = 0.0362 + 0.0064 \times (\text{mg } CO_2 \text{ evolved}).$$

TABLE 4. Mean incidence of infection of second-growth *Pseudotsuga menziesii* by *Armillaria ostoyae* 10 yr after silvicultural treatment and 1 yr after inoculation with isolates JR 1953 and DCI

Treatment	Block disease		Plug disease		Combined disease	
	%[a]	Rating[b]	%	Rating[b]	%	Rating[c]
Thinned (TT)	20 a	0.4 a	18 a	0.4 a	18 a	0.8 b
Thinned and fertilized (TF)	30 b	0.8 b	27 b	0.6 b	28 b	1.4 a
Unthinned control (UT)	18 a	0.6 a	16 a	0.4 c	17 a	1.0 c

[a] Disease percentages required natural log transformations to normalize data.

[b] Disease was rated as follows: 0, fungus not recovered; 1, fungus recovered from one inoculation site; 2, fungus recovered from two sites. Values are means of 60 samples (two isolates per tree × 10 trees × three sites). Within each column, values followed by the same letter are not significantly different at $P \leq 0.05$, as determined by Tukey's honest significant difference test.

[c] Incidence of infection is block incidence of infection plus plug incidence of infection. Values means are of 120 samples (four inoculations per tree × 10 trees × three sites). Within each column, values followed by the same letter are not significantly different at $P \leq 0.05$, as determined by Tukey's honest significant difference test.

DISCUSSION

Incidence of infection for the individual methods of inoculation, block, plug, and both methods combined (total) was highest in the TF treatment. The fungus also grew more rapidly on root bark tissue or on medium amended with root bark extracts from trees in TF stands. Thus, trees in TF stands appear to be more susceptible to infection by *A. ostoyae* than do trees in TT stands. However, we found no difference in wood production per square meter of leaf area per year between the TF and TT treatment. Less vigorous trees in the Northeast are more susceptible to infection by *Armillaria* spp. (49). Trees that are growing extremely fast may allocate more carbon to sugar and cellulose and less carbon to tree defense compounds, such as lignin, phenolics, and tannins. Trees that are highly stressed photosynthesize very little (4) and may not allocate an adequate amount of carbon to supply the shikimic acid pathway, which produces defense compounds. Stress also alters nitrogen metabolism and concentrations of the amino acids asparagine and proline in root bark (45), and defoliation and drought increase the overall concentration of nitrogen in root bark tissue of trees (35).

Trees with root bark containing a higher concentration of lignin and phenolic compounds had a lower incidence of infection by *A. ostoyae*. Trees with root bark containing a higher concentration of sugar had a higher incidence of infection by *A. ostoyae*. Lignin, tannins, and phenolics are synthesized by the shikimic acid pathway (20). Plants that are growing extremely fast, or otherwise are limited in carbon production, preferentially may allocate carbon to primary cell wall development, producing such compounds as hemicellulose and cellulose rather than shikimic acid products. High amounts of nitrogen may shift carbon allocation from the shikimic acid pathway to sugars to support increased photosynthetic area, thereby reducing concentrations of phenolics and lignins in root bark tissue. Nitrogen fertilization combined with thinning did increase leaf area and stem growth of these trees relative to the TT treatments. Study trees were not deficient in any nutrient measured. Trees supplied with additional nitrogen were able to assimilate the other necessary nutrients from the soil.

Results of our study are similar to those of others who have analyzed phenolic concentrations in plant tissues. Bryant et al (6) reported that increased N fertilization increased tree growth but decreased the concentration of phenolic compounds in leaf tissue of *Betula resinifera*. Palo et al (34) found that the concentration of phenolic compounds in leaf tissue of *B. pendula* decreased as growth increased, but rose again when growth slowed.

TABLE 5. Coefficient of determination (r^2) of infection of second-growth *Pseudotsuga menziesii* by *Armillaria ostoyae* with biochemical parameters measured in the bark of roots (0.5–3.0 cm diameter) 10 yr after silvicultural treatment and 1 yr after inoculation[a]

Parameter	Incidence of block infection		Incidence of plug infection		Total incidence of infection	
	Linear	Curvilinear	Linear	Curvilinear	Linear	Curvilinear
Sugar	0.47*	0.63*	0.26*	0.27*	0.37*	0.38*
Starch	0.43*	0.43*	0.05	0.10	0.06	0.07
Cellulose	0.05	0.25	0.13	0.13	0.22	0.23
Lignin	0.07	0.07	0.53*	0.53*	0.57*	0.57*
Total extractable phenolics	0.05	0.25	0.10	0.13	0.17	0.18
Protein-precipitable tannins	0.05	0.47*	0.02	0.03	0.11	0.11
Phenolic/sugar ratio	0.36*	0.51*	0.25	0.26	0.34*	0.39*
$E_{pd}:E_{as}$[b]	0.42*	0.34*	0.31*	0.46*	0.12	0.77*
$E_{ld}:E_{as}$[b]	0.37*	0.47*	0.43*	0.55*	0.26	0.70*

[a] Sample size = 27.

[b] E_{pd} is the energy required to degrade phenols; E_{ld}, energy required to degrade lignin; E_{as}, energy available from sugars.

[c] Asterisk indicates a correlation that is significantly different from a straight line drawn through the data points ($P \leq 0.01$).

The results of the experiment reported here are also consistent with those reported by Wargo (47). The major changes in roots induced by stress occur among the carbohydrates (45,48). Concentrations of starch and sucrose in the bark and outerwood of the roots are decreased, whereas concentrations of glucose and fructose are increased. Glucose enables the fungus to grow more rapidly in the presence of phenolic compounds (46).

Thinning stands of *P. menziesii* may be an important silvicultural procedure to keep trees physiologically resistant to attack by *A. ostoyae*. Davidson and Rishbeth (12) reported that suppressed *Pinus sylvestris* and *Quercus robur* were preferentially infected by *A. ostoyae* and *A. mellea*. Seedlings from five species of western conifers grown with balanced light and nutrients were significantly less susceptible to infection by *A. ostoyae* than were seedlings grown with limited light or nitrogen (15). In the experiment reported herein, trees in the UT stands were subject to competition for light because of crown closure; however, they were not suppressed.

Fungi can degrade phenolic compounds only when an additional carbon source is present; the rate of degradation is directly proportional to the amount of additional growth substrate (29). Carbon utilization by *A. ostoyae* in culture was more efficient at low sugar concentrations, but fungal biomass was greater at higher sugar concentrations. In forest environments, greater fungal biomass may indicate a greater ability of the pathogen to degrade phenolics and colonize host trees. The low sugar concentrations in the roots of trees growing in TT stands may have provided less energy for *A. ostoyae* to degrade phenolic compounds and lignin.

The correlation of E_{pd}:E_{as} and E_{ld}:E_{as} with total incidence of disease indicates that these parameters may provide dependable assays of the physiological response of trees to attack by *Armil-*

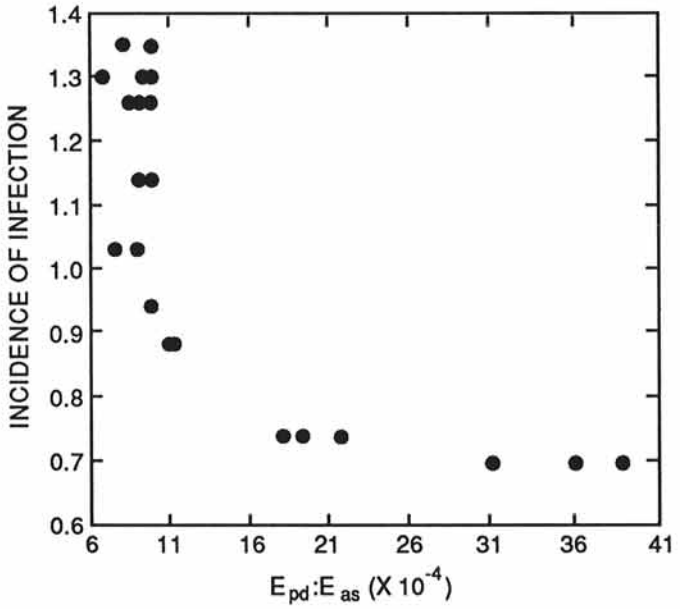

Fig. 1. Plot of the relationship of E_{pd}:E_{as} (ratio of energy required for phenolic degradation: energy available from sugars) to *Armillaria ostoyae*, combined incidence of infection ($r^2 = 0.77$, $P \leq 0.01$). Incidence of infection $= -2.029 + [(E_{pd}:E_{as})(4.840 \times 10^{9})] + [(E_{pd}:E_{as})^2(-2.771 \times 10^{18})] + [(E_{pd}:E_{as})^3(6.548 \times 10^{-26})]$.

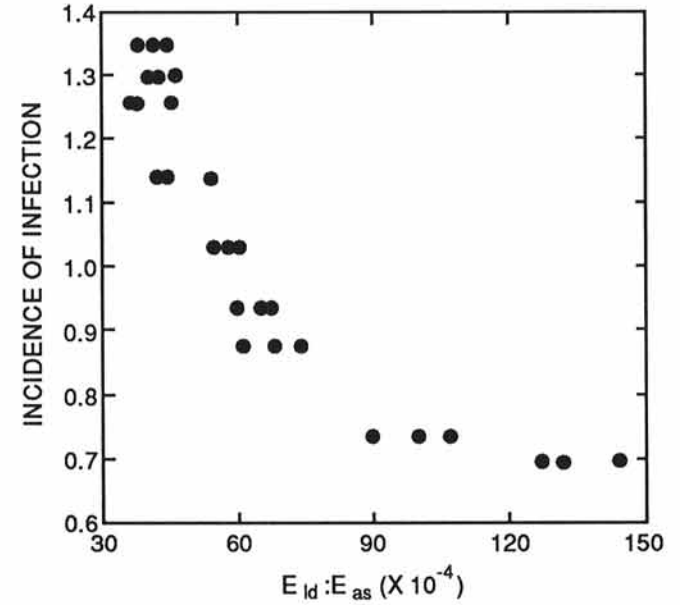

Fig. 2. Plot of the relationship of E_{ld}:E_{as} (ratio of energy required for lignin degradation: energy available from sugars) to *Armillaria ostoyae*, combined incidence of infection. ($r^2 = 0.70$, $P \leq 0.01$). Incidence of infection $= 0.803 + [(E_{ld}:E_{as})(-2.23 \times 10^{5})] + [(E_{ld}:E_{as})^2 (4.20 \times 10^{11})] + [(E_{ld}:E_{as})^3 (-5.02 \times 10^{-22})]$.

TABLE 6. Hyphal growth and mycelial dry weight of *Armillaria ostoyae* grown in Melin-Norkrans medium amended with phenolics extracted from root bark[a,b]

	Hyphal growth (mm)			Mycelial weight (mg)
Treatment[c]	3 wk	6 wk	9 wk	9 wk
Control	16.0 a	27.6 b	38.8 b	119.3 c
Thinned				
Phenol/glucose				
0.5:1.0	12.2 a	32.9 a	47.9 a	125.6 b
1.0:1.0	0.2 b	2.6 e	5.8 f	16.2 g
1.5:1.0	0.0 b	0.2 f	1.6 g	4.6 h
Thinned plus fertilized				
Phenol/glucose				
0.5:1.0	17.4 a	35.4 a	52.0 a	143.4 b
1.0:1.0	5.8 c	14.4 c	22.1 d	65.5 e
1.5:1.0	0.6 cb	19.7 c	28.4 c	82.8 d
Unthinned				
Phenol/glucose				
0.5:1.0	17.9 a	37.5 a	57.5 a	160.9 a
1.0:1.0	8.6 b	19.7 c	28.4 b	82.8 d
1.5:1.0	3.9 c	10.3 c	15.3 e	44.0 f

[a] Values are means of nine samples (root bark from three trees × three sites; each value is the mean of five inoculations). Within each column, values followed by the same letter are not significantly different at $P \leq 0.05$, as determined by Tukey's honest significant difference test.

[b] Phenolics were extracted from the bark of roots (0.5–23.0 cm in diameter) of second-growth *Pseudotsuga menziesii* 10 yr after silvicultural treatment.

[c] Ratio of gram of root bark phenolic equivalents/gram of glucose. Control medium was not modified.

TABLE 7. Evolution of carbon dioxide from colonies of isolates DCl, JR 1953, and TY 186 of *Armillaria ostoyae* after 3, 6, and 9 wk of growth on ground root bark of *Pseudotsuga menziesii* [a,b,c]

	CO_2 evolved (mg CO_2 gm^{-1} dry weight)		
Treatment	3 wk	6 wk	9 wk
Thinned (TT)	0.508 a	0.608 a	0.648 a
Thinned and fertilized (TF)	0.532 a	0.734 c	0.759 c
Unthinned (UT)	0.557 a	0.647 b	0.712 b

[a] Values are means of 27 observations (root bark tissue from three trees × three sites × three fungal isolates; each is the mean of five inoculations) expressed in terms of dry weight of root bark. Within each column, values followed by the same letter are not significantly different at $P \leq 0.05$, as determined by Tukey's honest significant difference test.

[b] Bark was from roots (0.5–3.0 cm in diameter) of second-growth *Pseudotsuga menziesii* 10 yr after silvicultural treatment.

[c] There was no significant isolate × stand interaction, or among the three isolate interactions. Therefore, data are presented with regard to stand treatment.

laria. The equations would be more accurate if the identities of the phenolic compounds in root bark of trees and the biological pathways of phenolic degradation by *Armillaria* were elucidated. The E_{pd} and E_{ld} we used are based on the pathway of lignin degradation by fungi (7), particularly *Phanerochatae chrysosporium* (29). Fungi convert most phenolic compounds to catechol or protocatechuic acid before ring fission occurs. We could not include the energy requirements necessary for these conversions, because the specific identities of each phenolic compound are unknown and the energy requirements of demethylation and decarboxylation vary greatly with each specific phenolic molecule. These energy requirements are relatively small when compared with the energy required for cleavage of a benzene ring.

When reforesting a site infested with *Armillaria*, land managers should consider inoculum potential as a significant factor in making silvicultural decisions (39). Commercial thinning may produce trees that are more resistant to *A. ostoyae*. This effect could be negated by the possible increase in infections resulting from fungal colonization of the residual stumps. The financial cost of the previous recommendations, i.e., use of commercial thinnings to reduce *Armillaria* infections, may not be warranted. Thinning increases dead root biomass, which acts as an energy source for the fungus and, therefore, can increase the incidence of the disease (17,33). If stands of *P. menziesii* are thinned to the desired spacing when trees are small, biomass of dead roots is less than when large trees of commercial size are thinned. Potential inoculum is diminished and invasion by *A. ostoyae* can be minimized. In these situations, thinning can be an effective method to control infection (25,40).

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