Ethanol synthesis by anoxic root segments from five cedar species relates to their habitat attributes but not their known differences in vulnerability to *Phytophthora lateralis* root disease

Rick G. Kelsey, Gladwin Joseph, and Michael G. McWilliams

Abstract: Ethanol synthesis by anoxic root segments from Port Orford cedar (*Chamaecyparis lawsoniana* (A. Murray bis) Parl.); yellow cedar (*Chamaecyparis nootkatensis* (D. Don) Spach); Atlantic white cedar (*Chamaecyparis thyoides* (L.) Britton, Sterns & Poggenb.); western redcedar (*Thuja plicata* Donn ex D. Don), and incense cedar (*Calocedrus decurrens* (Torr.) Florin) was compared to determine whether the amounts that they produced during flooding could contribute the known greater vulnerability of Port Orford cedar to infection by *Phytophthora lateralis* Tucker & Milbrat. Roots were incubated in water at 5, 15, 25, and 35 °C for 14 days with periodic sampling. After 12 h of anoxic stress, Atlantic white cedar and yellow cedar roots produced equal quantities of ethanol that were about two times more than produced by the other three species, which did not differ from one another. The roots remained anoxic for 14 days, with ethanol concentrations increasing 6 to 11 times depending on the species. After 14 days, Atlantic white cedar remained the highest ethanol producer at two to three times more than the other species, whereas incense cedar yields were the lowest. Yellow cedar, western redcedar, and Port Orford cedar had intermediate levels of ethanol. The similarity in responses of Port Orford cedar to the other species is strong evidence that ethanol is not an important contributor to its known greater vulnerability to *P. lateralis* infection. In general, root incubation temperature affected ethanol synthesis similarly for all species. Increases in temperature from 5 to 15 °C or 15 to 25 °C doubled the ethanol yields at 12 h. Literature ratings of anaerobic tolerance for these cedars were compared with ratings based on their ethanol yields after 12 h or 14 days of anoxia. The latter rating appears to more closely correspond with the cedars associations to wet, mesic environments and their likelihood of experiencing anoxia via flooding.

Résumé : La synthèse d'éthanol par des segments de racines en conditions anoxiques, provenant du faux-cyprès de Lawson (*Chamaecyparis lawsoniana* (A. Murray bis) Parl.), du faux-cyprès de Nootka (*Chamaecyparis nootkatensis* (D. Don) Spach), du faux-cyprès blanc (*Chamaecyparis thyoides* (L.) Britton, Sterns & Poggenb.), du thuya géant (*Thuja plicata* Donn ex D. Don) et du calocèdre à encens (*Calocedrus decurrens* (Torr.) Florin), a été comparée pour déterminer si la quantité d'éthanol produit par les racines lorsqu'elles sont inondées pouvaient contribuer au fait que le faux-cyprès de Lawson soit plus sensible à l'infection par *Phytophthora lateralis* Tucker & Milbrath. Les racines ont été incubées dans l'eau à 5, 15, 25 et 35 °C pendant 14 jours et échantillonnées périodiquement. Après 12 h de stress anoxique, les racines du faux-cyprès blanc et du faux-cyprès de Nootka produisaient des quantités égales d'éthanol qui étaient environ deux fois plus élevées que chez les autres essences qui ne différaient pas les unes des autres. Les racines sont demeurées en conditions anoxiques pendant 14 jours et les concentrations d'éthanol ont augmenté de 6 à 11 fois selon l'essence. Après 14 jours, le faux-cyprès blanc est demeuré l'espèce qui produisait le plus d'éthanol, soit deux à trois fois plus que les autres essences, tandis que la production du calocèdre à encens était la plus faible. La production d'éthanol par le faux-cyprès de Nootka, le thuya géant et le faux-cyprès de Lawson était intermédiaire. La similitude entre la réponse du faux-cyprès de Lawson et celle des autres essences constitue une preuve solide que l'éthanol n'est pas responsable de la plus grande sensibilité de cette essence à l'infection par *P. lateralis*. En général, la température d'incubation des racines a eu un effet similaire sur la synthèse d'éthanol chez toutes les essences. Des augmentations de 5 à 15 °C ou de 15 à 25 °C ont doublé la production d'éthanol après 12 h. Le classement qui est rapporté dans la littérature selon la tolérance à l'anoxie de ces essences a été comparé au classement basé sur leur production d'éthanol après 12 h ou 14 jours en conditions anoxiques. Ce dernier classement semble correspondre davantage à l'association de ces essences à des milieux humides ou mésiques et à leur chance d'être placées en conditions anoxiques à cause des inondations.

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Introduction

Yellow cedar, western redcedar, incense cedar, and Port Orford cedar (see Table 1 for nomenclature) all grow in the temperate coniferous forests of western North America, with Oregon being the only state in which all four are native. Port Orford cedar is the most restricted geographically, limited to southwestern Oregon and northwestern California (Zobel et al. 1985; Zobel 1990). It is valued for its ecological importance (Goheen 2000; Jimerson et al. 2003) and wood properties such as straight grain and decay resistance. Its greatest commercial value has been in Asian markets where it is highly prized as a replacement for Japanese hinoki cedar, *Chamaecyparis obtusa* (Siebold & Zucc.) Endl. (Barnes and McLean 2003). Port Orford cedar typically grows as scattered individuals or in small noncontiguous stands across a diversity of habitats. Although noted for its association with ultramafic soils, it is common along waterways or sites where year-round seepage can provide a consistent summer water supply (Zobel et al. 1985; Hansen et al. 2000).

Phytophthora lateralis is an introduced pathogen of unknown origin that causes a fatal root rot and strongly limits opportunities to maximize ecosystem services from Port Orford cedar (Zobel et al. 1985; Hansen et al. 2000; Goheen et al. 2003). This pathogen was initially discovered in the early 1920s on nursery stock and, by 1952, had spread into the native stands of Port Orford cedar in southwestern Oregon (Zobel et al. 1985; Hansen et al. 2000). This essentially ended the horticultural use of this species for landscaping and currently continues to threaten native populations (Hansen et al. 2000; Goheen et al. 2003).

Port Orford cedar and Pacific yew, *Taxus brevifolia* Nutt., are the only two confirmed hosts of *P. lateralis* in native forests, with the latter being much more resistant (DeNitto and Kliejunas 1991; Murray and Hansen 1997). The other three western cedars and Atlantic white cedar, a native of Atlantic and Gulf coast forests (Table 1), appear to be nearly immune. In seedling experiments, Atlantic white cedar and yellow cedar remained alive for 2 years after inoculation with *P. lateralis* compared with 96% mortality for Port Orford cedar seedlings (Hunt and O'Reilly 1984). A low level of mortality (<10%) was confirmed for yellow cedar seedlings or grafted seedlings with yellow cedar rootstock planted on infested forest sites (McWilliams 2000). Infection of yellow cedar under natural conditions has not been demonstrated and is probably uncommon as there is limited range overlap with Port Orford cedar where *P. lateralis* inoculum would be most abundant. Although most Port Orford cedar are highly susceptible to *P. lateralis*, trees with natural resistance have been identified and incorporated into an active breeding program to improve resistance (Hansen et al. 1989; Sniezko 2006; Oh et al. 2006).

Zoospores and chlamydospores are the key means of *P. lateralis* dispersal (Goheen et al. 2003). The vegetative chlamydospores are not motile and are relatively resistant to damage from drying or temperature, allowing them to survive for up to 7 years in infected tree tissue or fragments of tissue in the soil (Hansen and Hamm 1996). These infested materials are readily transported to healthy trees or stands by animals, humans, or equipment (Hansen et al. 2000; Goheen et al. 2003). Alternatively, zoospores are released from sporangia into water and propelled by two flagella, allowing them to move short distances, settle, and encyst on host roots (Carlile 1983; Deacon 1996). They will also encyst if agitated in water or after a period of time if they fail to encounter host roots. Running water helps transport zoospores and cysts long distances (Goheen et al. 2003). Port Orford cedar habitats are cool and wet during late fall, winter, and early spring, with mean monthly soil temperatures at 20 cm depth ranging between 1 and 12 °C, depending on the site (Zobel and Hawk 1980). This wet, cool environment is especially suited for zoospore production, distribution, germination, and growth in the host (Trione 1974; Goheen et al. 2003).

In addition, during this cool, rainy season, there is a higher likelihood for brief periods of root flooding for Port Orford cedar trees on sites with elevated water tables or adjacent to waterways. When plant roots are submerged in water, their O_2 supply is blocked and internal O_2 will decrease to hypoxic ($O_2 < 20.9\%$ but $> 0\%$) or anoxic ($O_2 = 0\%$) levels as metabolically active cells continue to respire. As the tissue O_2 is depleted, biochemical processes are initiated that bring about major changes including (*i*) a dramatic reduction in high energy consuming metabolic processes and (*ii*) a switch in the production of ATP energy from mitochondrial oxidative phosphorylation to cytoplasmic fermentation, where a much reduced level of ATP energy is generated by glycolysis in conjunction with ethanol synthesis (Gibbs and Greenway 2003; Greenway and Gibbs 2003; Bailey-Serres and Voesenek 2008, Magneschi and Perata 2009). This fermentation energy is used to maintain critical membrane integrity, selectivity, and function needed to prevent cellular acidity from reaching lethal levels and allows the cells and tissues to remain alive for short periods until O_2 supplies can be replenished when flooding subsides (Greenway and Gibbs 2003; Gibbs and Greenway 2003; Felle 2005). If flooding persists for extended periods, some plant species have adapted other survival mechanisms allowing them to escape or endure (Crawford 1989; Kozlowski 1997; Parolin et al. 2004; Glenz et al. 2006; Bailey-Serres and Voesenek 2008). However, the roots of species without these long-term options can only remain alive until the carbohydrate pool used for glycolytic ATP production is depleted, then the membranes fail, acidosis resumes, and the roots die (Crawford 2003; Gibbs and Greenway 2003; Greenway and Gibbs 2003; Felle 2005). Of the five cedar species in this study, Atlantic white cedar is recognized as the most tolerant of anaerobic environments (U.S. Department of Agriculture Natural Resources Conservation Service (USDA NRCS) 2009, The PLANTS Database, http://plants.usda.gov/java).

Soon after root ethanol synthesis is initiated, it begins to diffuse, readily passing through membranes, with some moving up the stem transpirational stream and some being lost to the surrounding flood water (Crawford and Finegan 1989; Joseph and Kelsey 1997; Kreuzwieser et al. 1999). Ethanol exuding from flooded roots, either alone or in combination with other root exudates such as sugars, amino acids, fatty acids, or flavonoids, can function as attractants for zoospores of oomycetes including various *Phytophthora* species, although *P. lateralis* remains to be studied (Halsall 1976; Carlile 1983; Leaño et al. 1998; Tyler 2002). The amount of ethanol synthesized by flooded conifer roots can differ tremendously among species (Crawford and Baines 1977).

Table 1. Ranges and select habitat attributes of the five cedar species in this study.

Species	Range and habitat attributes
Atlantic white cedar (AWC) (*Chamaecyparis thyoides* (L.) Britton, Sterns & Poggenb.)	Range: Atlantic coast from Maine to northern South Carolina; northwest Florida along Gulf coast into Mississippi Elevation: low coastal, typically 1–43 m Precipitation: 1020–1630 mm Soil–site: wet muck (peat), bogs or swamps, near standing water, low nutrients, acidic (pH often 3.5–5.5) Source: Little and Garrett 1990
Yellow cedar (YC) (*Chamaecyparis nootkatensis* (D. Don)) Spach)	Range: Pacific coast of Southeast Alaska and western Canada, Cascade ranges of Washington and Oregon Elevation: variable, 4–2300 m Precipitation: 2130–2340 mm Soil–site: most abundant and dominant on wet and boggy sites with shallow water table, often nutrient poor and thin organic soils, rich in calcium and magnesium, (pH 4.2–7.5); also does well on deep, well-drained soils. Source: Harris 1990; D'Amore et al. 2009
Western redcedar (WRC) (*Thuja plicata* Donn ex D. Don)	Range: Pacific coast forests of Southeast Alaska and western British Columbia, south throughout the Coast and Cascade ranges of Washington and Oregon; interior forests of British Columbia, western Montana, and northern Idaho. Elevation: variable, 1–2290 m coastal; 320–2130 m interior Precipitation: 890–6600 mm coastal; 710–1240 mm interior Soil–site: most abundant on wet and boggy sites with shallow water table, dominates wet ravines and poorly drained depressions; often nutrient poor, (pH 5.1–7.1); also does well on deep, well-drained soils. Source: Minore 1990; D'Amore et al. 2009
Port Orford cedar (POC) (*Chamaecyparis lawsoniana* (A. Murray bis) Parl.)	Range: Pacific coast forests of southwestern Oregon and the northwestern corner of California, with isolated inland populations near Mt. Shasta Elevation: variable, 20–1950 m Precipitation: 1000–2250 mm Soil–site: variable, sandy to clay, dominant on wet soils, drainage ways, or near seepage, acidic (pH 4.2–7.0) Source: Zobel et al. 1985; Zobel 1990
Incense cedar (IC) (*Calocedrus decurrens* (Torr.) Florin)	Range: Cascades in northern and central Oregon, Coast Range and Siskiyou Mountains of southern Oregon and northern California, Sierra Nevada Mountains of northern and central California, sporadic in southern California and Baja California Elevation: variable, 50–2960 m Precipitation: 380–2030 mm Soil–site: variable, cool–moist to hot–dry; coarse sand to fine clay (pH 4.7–7.1) Source: Powers and Oliver 1990

Note: Nomenclature for yellow cedar is currently in dispute as either *Xanthocyparis nootkatensis* or *Callitropsis nootkatensis* (Mill and Farjon 2006; Little 2006). Yellow cedar, western redcedar, and incense cedar soil pH values are from the U.S. Department of Agriculture Natural Resources Conservation Service (2009) PLANTS Database.

Thus, if ethanol production by anoxic Port Orford cedar roots is sufficiently higher than the other cedars, it could contribute to its greater vulnerability to *P. lateralis*. The primary objective of this project was to measure the synthesis of ethanol over time for segments of Port Orford cedar roots submerged in water and compare their response with root segments from the other three western cedar species and Atlantic white cedar.

All of the biochemical changes in response to anaerobic stress are enzymatically controlled and strongly influenced by temperature. Although Port Orford cedar grows across the same elevational gradient as the other three western cedars in this study, its geographic range is much more restricted (Table 1) and soils remain relatively cool year round from the influence of nearby water. Alternatively, over parts of their ranges, both Atlantic white cedar and incense cedar grow on sites with warmer soils, especially during summer. Thus, a second objective was to determine whether incubation temperatures of the root segments, especially at cooler temperatures typical of winter Port Orford cedar environments when infection is more likely, influenced ethanol synthesis differently among species.

Materials and methods

Seed source

Seeds of all species were obtained from open-pollinated trees. Port Orford cedar and western redcedar were from separate, single-seed zones in Oregon, whereas incense cedar was collected at two zones in California. Yellow cedar seed originated from three zones in Washington and two in Oregon (McWilliams 2000), whereas the Atlantic white cedar was collected in stands from Burlington County, New Jersey.

Seedling propagation

All seeds were sown and grown in tube containers for

1 year at the USDA Forest Service Dean Creek Nursery near Reedsport, Oregon, except for yellow cedar, which was grown by the Cowician Lake Research Station on Vancouver Island, British Columbia, Canada. Single, 1-year-old seedlings were transplanted into 2.6 L plastic pots containing a soilless mix of perlite (0.227 m^3), peat (0.227 m^3), and hemlock bark (~0.396 m^3), supplemented with Osmocote fertilizer (5 kg), Micromax micronutrients (1 kg), gypsum (agricultural, 1 kg), and 0–25–0 (10) superphosphate with sulfur (1 kg). Seedlings were kept outside at Oregon State University for 2 years and watered regularly with an automated irrigation system. This adaptation period was considered adequate to ameliorate any differences between yellow cedar and the other species that might have arisen from dissimilar growing conditions at their respective nurseries during the first year. While growing outside, they were treated with Chipco fungicide to prevent infection from *Botrytis cinerea* and with Ornalin and Talstar to prevent damage from root weevils. The seedlings were 3 years old when this experiment was initiated. Prior to sampling in mid-February, the seedling root systems had ample exposure to cool temperatures similar to Port Orford cedar environments. During the 120 days before sampling, there were 101 days when the minimum temperature was 5 °C or less, including 27 days when the maximum temperature never exceeded 5 °C and an additional 47 days when the temperature never exceeded 10 °C.

Seedling processing

Prior to sampling, the seedlings were numbered and randomly selected to determine their order for processing. One seedling of each species was processed per day over the course of eight days, yielding eight replicates. On each day at approximately 0800 h, the five selected seedlings were transferred to a coldroom (2–3 °C) where their stem diameter (2.0 cm above the soil with a caliper to the nearest 0.01 mm) and height (to the nearest 0.5 cm) were measured. The seedling was then removed from the pot and a subsample (25–50 g) of the fibrous roots was clipped free and washed in a cold water bath to dislodge media particles. Cleaned roots were rinsed with a 5% Chlorox solution for 60–90 s to sterilize the surface tissue and then washed with multiple rinses of cold distilled water. Excess water was removed by patting the roots with paper towels, and any remaining pieces of the soil media or obviously dead root fragments were discarded. The roots were clipped into pieces 1–3 cm long, which were placed in a plastic bag and stored on ice until one individual from each species had been processed for that day. They were then prepared for incubation.

Root preparation and incubation

About 3–4 g of root sample was weighed into a preweighed clear glass vial (22 mm × 75 mm, ~22 mL) containing three small glass beads (3 mm diameter) to assist in mixing the water solution. The vial was then filled with cold distilled water previously saturated with CO_2 (pH 4.0), leaving a small air space to prevent water loss when sealed with a preweighed Teflon-lined silicone septum. After a final weighing, the vial was stored on ice. Four root subsamples were prepared per species and placed into incubators set at 5, 15, 25, and 35 °C (one subsample of each species for each incubation temperature).

To estimate the root water content, two additional root subsamples were weighed into preweighed vials and oven dried for 16 h at 102 °C. The vials were then sealed and reweighed after adjusting to room temperature. Water content was calculated on a dry mass basis and averaged to give a single value per seedling for analysis. Another subsample of root tissue for nitrogen analysis was placed into a vial, which was heated at 100 °C for 1 h to kill the enzymes. The vial was then sealed and stored in a freezer (–36 °C) until all samples had been processed. After drying for 48 h at 70 °C, the vials were sealed and returned to the freezer for storage.

Ethanol analysis

A 5 μL sample of water was extracted with a syringe from each vial at 4, 8, 12, 24, and 336 h (14 days) and sealed in a headspace vial (22 mm × 75 mm, ~22 mL) with a Teflon-lined butyl rubber septum. The daily water supply was checked for ethanol (time = 0) with duplicate 5 μL samples. The ethanol content in all samples was analyzed by headspace gas chromatography as described in detail previously (Kelsey and Joseph 1998). Because these were water samples, they could be analyzed with a single gas chromatography run, rather than the multiple head space technique used for tissue samples. Three vials containing 5 μL of a standard ethanol–water solution were analyzed with each set of vials on the autosampler and used to calibrate the instrument. Ethanol concentrations were normalized with the water content measurements and reported as micromoles per gram of dry mass.

Nitrogen analysis

The dried root samples were ground in a Wiley mill to pass a 40-mesh screen and then were redried at 60 °C for 16 h prior to analysis. The total percentage N in the dried tissue was quantified with a Dumas combustion apparatus coupled with an isotope ratio gas chromatograph – mass spectrometer at the Stable Isotope Research Unit, Department of Soil Science, Oregon State University.

Statistical analysis

For all seedling parameters other than ethanol, the experimental design was a randomized complete block, with day representing block. Seedling height, diameter, percentage root nitrogen, and root water content were all analyzed with SAS software (version 9.2; SAS Institute Inc. 2008) using the MIXED linear model with species as treatment. Means were compared by Fisher's protected least significant difference (model $\alpha = 0.05$). Percentage root nitrogen and root water content required natural log transformation to meet normality or homogeneous variance requirements based on plots of the residuals. The regression of percentage root nitrogen vs. seedling height was done with PROC REG after removing two outliers.

The experimental design for ethanol concentrations at 12 h and 14 days was a randomized complete block, with a split plot (four temperatures per seedling) and repeated measures (two sample times). Log-transformed concentrations were analyzed by the MIXED linear model, with day representing block, and a UN(1) unstructured covariance because it provided the lowest AICC value of the nine covariance choices evaluated. Means were compared with Tukey–Kramer adjust-

ments. Transformed means and their 95% confidence limits were back-transformed to medians for presentation. Differences were considered significant at $P \leq 0.05$.

Results

After three years in pots, the species expressed some differences in height growth, with Port Orford cedar and western redcedar being the tallest and incense cedar being the shortest, although the latter was not significantly less than yellow or Atlantic white cedar (Fig. 1A). Stem diameters, however, were all the same (Fig. 1B), as were the root water contents (Fig. 1C). Nitrogen was measured because its availability can have a dramatic impact on the production and functioning of the enzymes that control cellular metabolism, including those regulating ethanol synthesis (Kelsey et al. 1998). Furthermore, most of these cedar species grow on nutrient poor sites (Table 1) where nitrogen may be limited, especially yellow cedar and western redcedar (D'Amore et al. 2009), and their uptake of readily available nitrogen could differ. Root nitrogen content was highest in incense cedar and yellow cedar (Fig. 1D) and lowest in western redcedar, Port Orford cedar, and Atlantic white cedar, with a moderate negative relationship to height growth (Fig. 1D, insert). The absence of any unusual responses from yellow cedar for any of these parameters supports the assumption that the two years of growth in pots was a sufficient adaptation period to minimize any residual impact from having spend the first year in a different nursery than the other species.

Changes in ethanol concentrations after 4, 8, 12, and 24 h of incubation are shown in Fig. 2. As expected, ethanol yields within species typically increased with each 10 °C rise in incubation temperature, except for some species at 24 h where the 25 and 35 °C roots produced the same quantities. Thus, 12 h ethanol yields were selected to represent the species response to brief periods of anoxic stress for statistical comparison with their response to an extended 14-day period of anoxic stress. In this analysis, all two-way interactions for species, time, and temperature were significant (all $P \leq 0.031$, Table 2), but not their three-way interaction ($P = 0.382$).

Species ethanol production over time (Fig. 3, averaged across temperatures) shows clustering into two groups at 12 h and three groups at 14 days incubation. At 12 h, Atlantic white cedar and yellow cedar produced the same levels of ethanol ($P = 1.000$) but significantly higher quantities (~two times) than the low-yielding group composed of incense cedar, Port Orford cedar, and western redcedar (all $P \leq 0.001$), with no differences among them (all $P \geq 0.947$). At 14 days incubation, Atlantic white cedar was the only member of the high group with two to three times more ethanol than the other four species (all $P \leq 0.002$). Yellow cedar had shifted from a high ethanol producer at 12 h to an intermediate producer at 14 days, with greater quantities than incense cedar ($P = 0.047$), which remained the only species in the low ethanol group. Although Port Orford cedar and western redcedar produced intermediate ethanol yields comparable with yellow cedar (all $P = 1.000$), they were not significantly greater than incense cedar ($P = 0.075$ and 0.156, respectively). The concentration increases after 14 days of flooding ranged from about 6 to 11 times the amounts at 12 h within

Fig. 1. Mean (±95% confidence limits) (A) seedling height, (B) stem diameter, (C) percentage root water content, and (D) percentage root nitrogen of the seedlings sampled for this experiment. The insert in part D shows the relationship between percentage root nitrogen and seedling height for the five cedars. Letters along the *x* axis in each graph indicate results of the statistical analyses. Those species with different letters along this axis are significantly different at $P \leq 0.05$. IC, incense cedar; YC, yellow cedar; AWC, Atlantic white cedar; WRC, western redcedar; POC, Port Orford cedar.

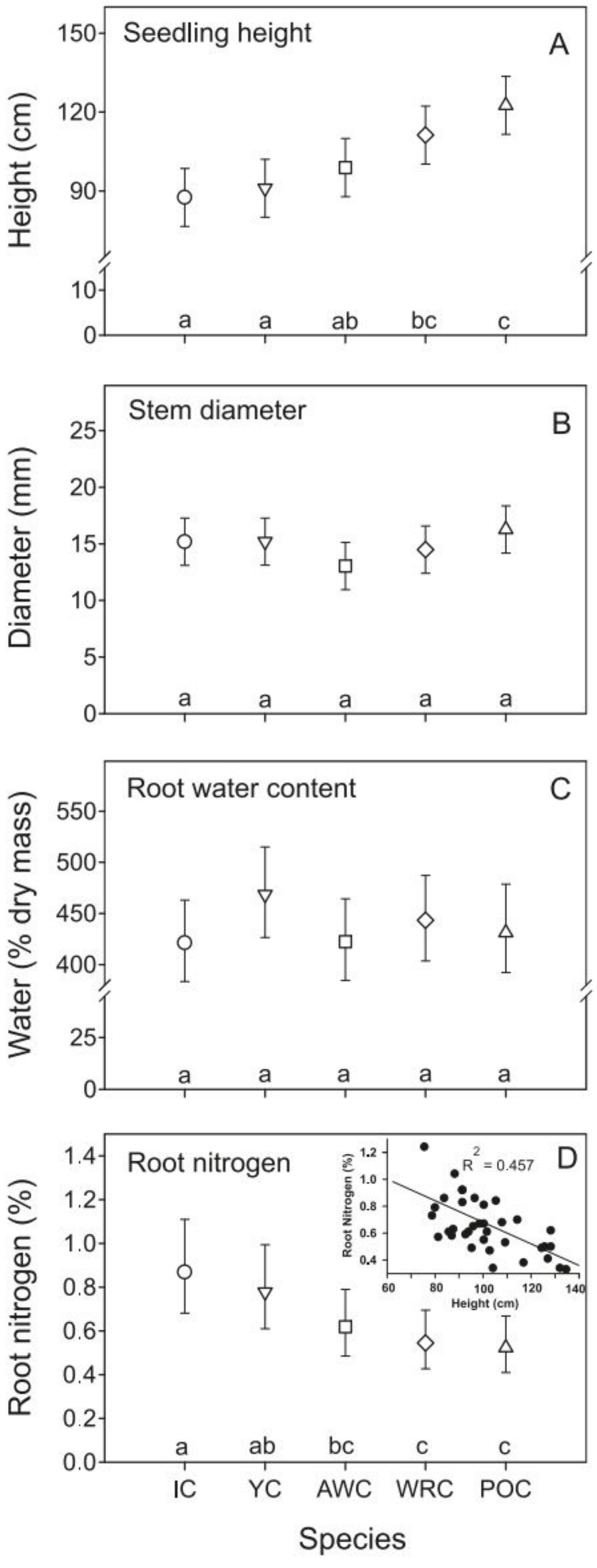
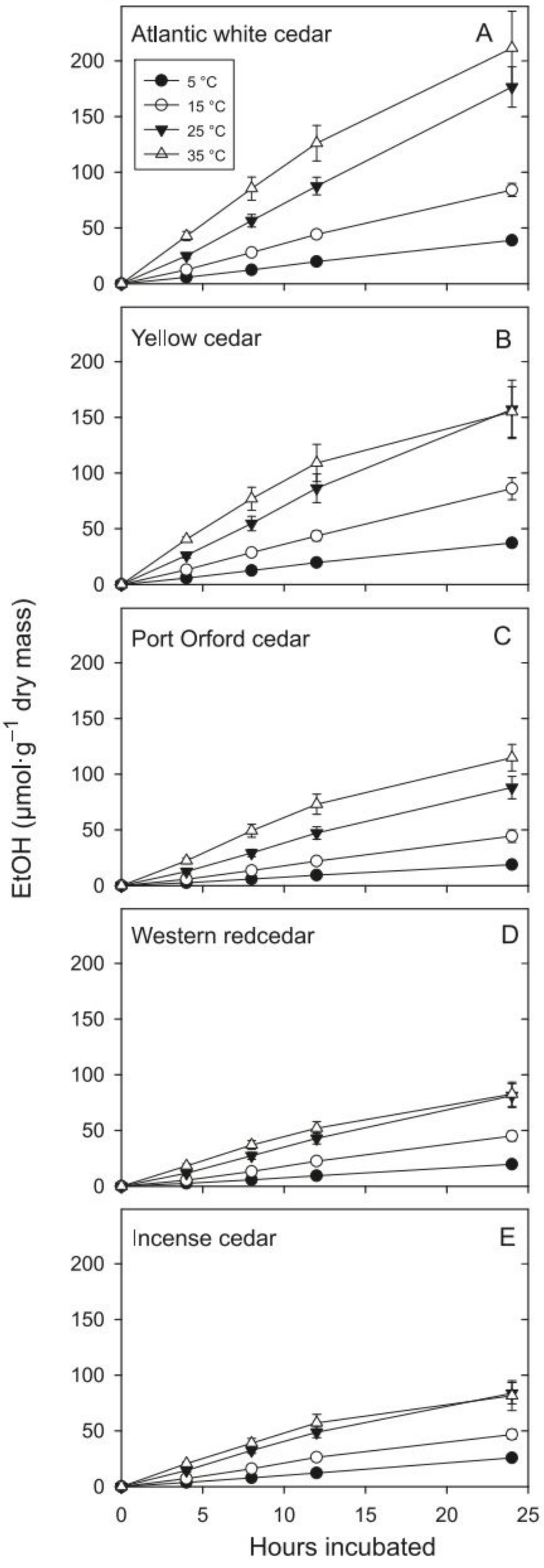


Fig. 2. Mean (±1 standard error) ethanol concentrations produced by root segments from five cedar species during 24 h of anoxic stress at four temperatures.



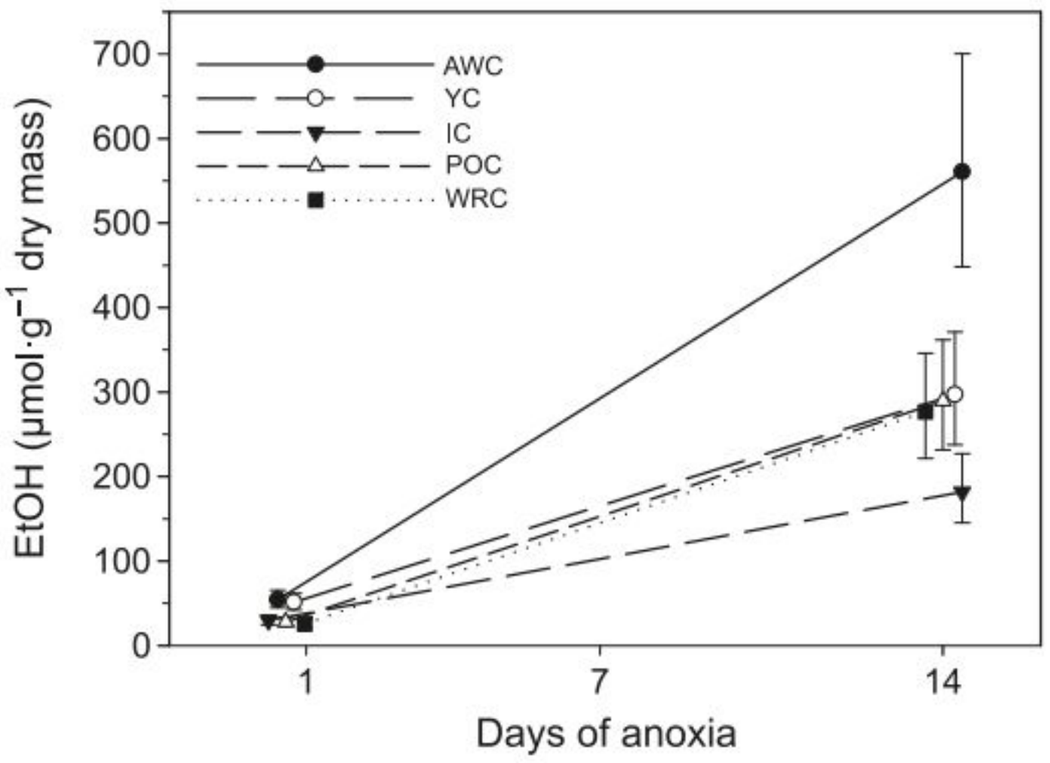
each species (Fig. 3), and all changes were significant (all $P < 0.000$). Species differences in ethanol synthesis appeared unrelated to root nitrogen concentrations, as western redcedar roots contained intermediate nitrogen levels (Fig. 1D) but produced the highest ethanol of all species, regardless of the anaerobic period (Fig. 3). Incense cedar produced the lowest ethanol at 14 days (Fig. 3) and its roots contained the highest quantities of nitrogen (Fig. 1D).

Table 2. Analysis of variance results for the effects of species, incubation temperature, and days of anoxic stress on ethanol concentrations produced by cedar root segments.

	df		
Effect	N	D	Probablility > *F*
Days (D)	1	140	<0.000
Species (S)	4	28	<0.000
Temperature (T)	3	105	<0.000
S × T	12	105	0.031
T × D	3	140	<0.000
S × D	4	140	<0.000
S × T × D	12	140	0.382

Note: df, degrees of freedom; N, numerator; D, denominator.

Fig. 3. Median (±95% confidence limits) ethanol concentrations (averaged across all five temperatures) produced by root segments from five cedar species after 12 h and 14 days of anoxic stress. Symbols are offset to improve view. See Table 1 for explanation of species codes.



Species responses to the four temperatures (Fig. 4, averaged across days of stress) were similar for Port Orford cedar, western redcedar, and incense cedar, with no differences among them at any temperature (all $P \geq 0.393$). In contrast, Atlantic white cedar produced greater quantities of ethanol than all three of them at each temperature (all $P \leq 0.033$), except for Port Orford cedar at 35 °C ($P = 0.151$). Yellow cedar concentrations fell between these two groups, but without significant differences from species in either group, at any temperature (all $P \geq 0.059$). Root incubation at 15 °C for all species yielded greater amounts of ethanol than at 5 °C ($P < 0.000$), whereas only Atlantic white cedar ($P \leq 0.008$) and Port Orford cedar ($P \leq 0.024$) yielded greater quantities at 25 °C than roots incubated at 15 °C. Port Orford cedar was the only species with roots incubated at 35 °C that produced greater ethanol yields than roots incubated at 15 °C ($P = 0.029$). Although concentrations at 35 °C were lower

Fig. 4. Median (±95% confidence limits) ethanol concentrations (averaged for the 12 h and 14 day incubation periods) produced by anoxic root segments from five cedar species subjected to four temperatures (°C). Symbols are offset to improve view. See Table 1 for explanation of species codes.

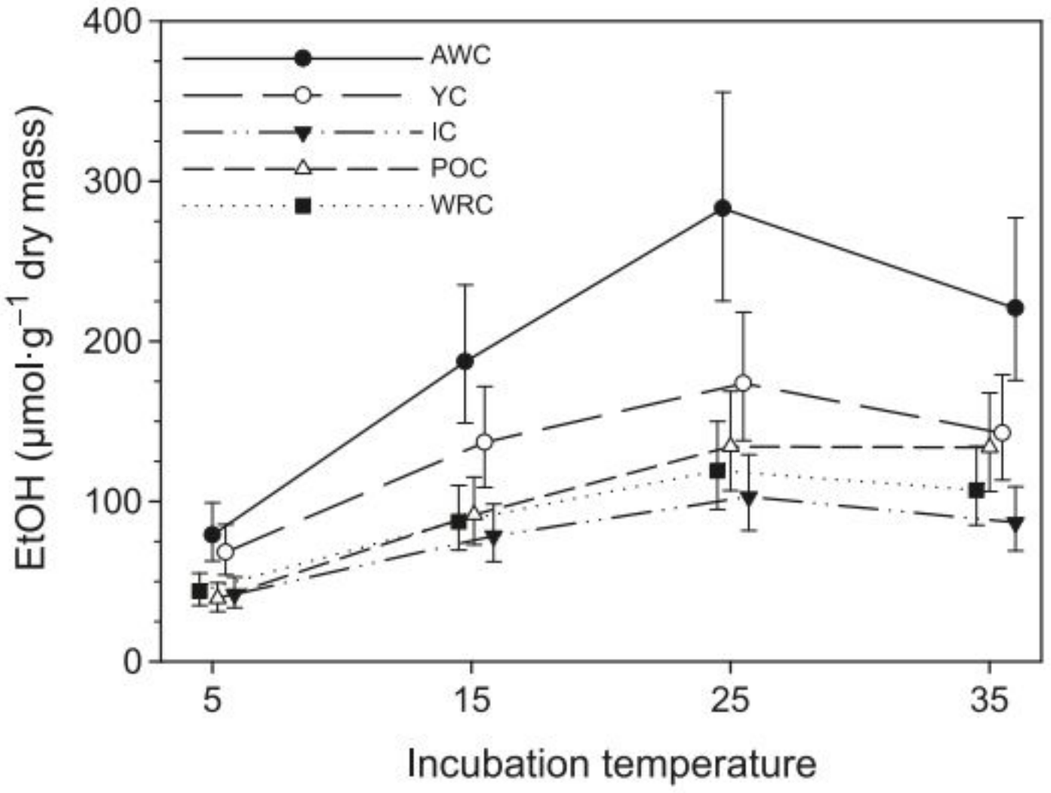
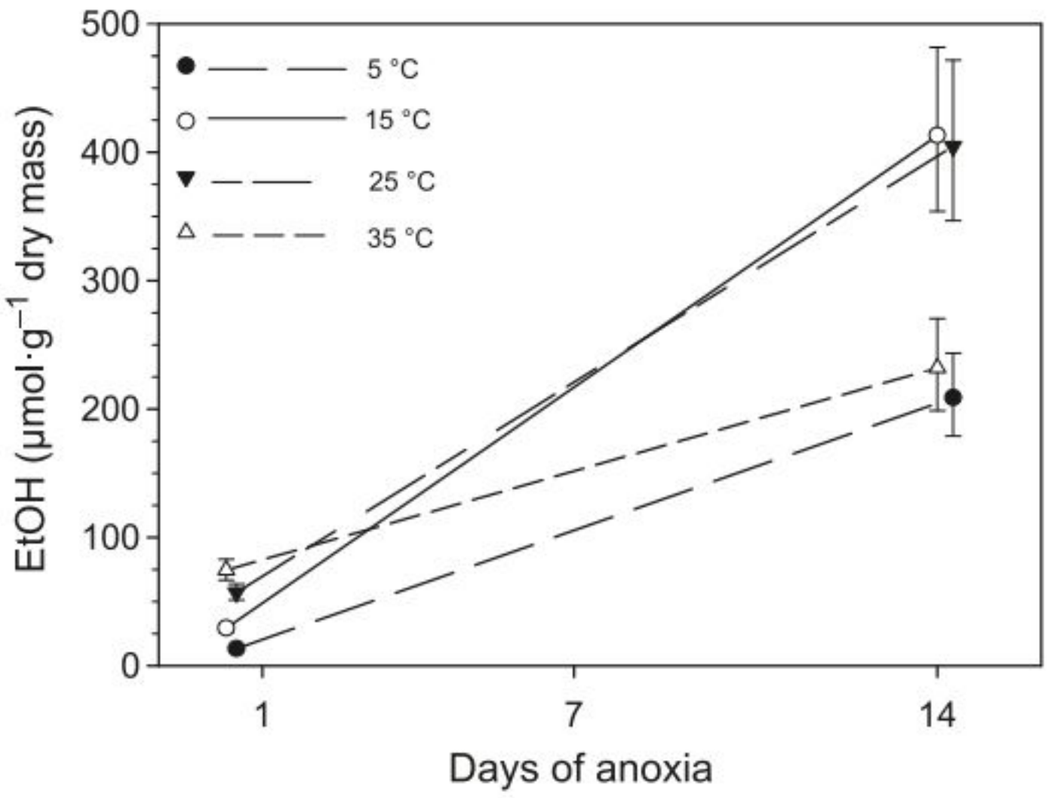


Fig. 5. Median (±95% confidence limits) ethanol concentrations (averaged across the five species) produced by anoxic root segments incubated at four temperatures for 12 h and 14 days. Symbols are offset to improve view.



than at 25 °C for all species (except Port Orford cedar), none of these differences was significant (all $P \geq 0.568$).

Incubation temperature over time did impact ethanol concentrations (Fig. 5), with the same response for all species. At 12 h incubation, ethanol yields were lowest at 5 °C and increased significantly with each additional 10 °C rise in incubation temperature (all $P < 0.000$). After 14 days of incubation, the roots incubated at 15 and 25 °C had the same ethanol yields ($P = 1.000$) but twice the yields of the roots incubated at 5 and 35 °C (all $P < 0.000$), which did not differ from one another ($P = 0.907$). The increases in ethanol between 12 h and 14 days were significant for each of the four incubation temperatures (all $P < 0.000$).

Discussion

Species comparisons must be interpreted with some caution as the seedlings originated from one to five seed zones and did not include sources from across their geographic range. However, the seed sources were selected without any preconditions and thus do provide insight into how the species may differ.

The species grouping from cumulative ethanol synthesis after an extended 14 days of anoxic stress was different from their grouping after a short 12 h period of anoxia (Fig. 3). Atlantic white cedar and incense cedar were the high and low ethanol producers, respectively, after both periods of stress. Although yellow cedar ethanol yields were high and comparable with Atlantic white cedar yields at 12 h, by 14 days, the yellow cedar ethanol levels had dropped to an intermediate amount similar to the levels in western redcedar and Port Orford cedar, which were initially low ethanol producers at 12 h. These relative changes in ethanol over time may result in part from species differences in available carbohydrate concentrations or the types and quantities of enzymes that they produce during anoxia to regulate carbohydrate metabolism (Bailey-Serres and Voesenek 2008). Concentrations of soluble sugars, glucose, fructose, and sucrose and their rates of metabolism were probably key factors in the 12 h ethanol yields, as in cereal grains (Guglielminetti et al. 2001), whereas the concentrations of starch and synthesis of α-amylase that degrades starch into glucose probably influenced the species changes in ethanol concentrations from 12 h to 14 days, as starch typically supplies glycolysis and fermentation with carbohydrates in other plants during extended anoxia (Ismail et al. 2009; Magneschi and Perata 2009; Harada and Ishizawa 2003).

Species responses to the four incubation temperatures were generally similar especially among the western cedars (Fig. 4). Atlantic white cedar was least similar as its magnitude of change in ethanol production with temperature was typically greater than that of the western species. The length of exposure to a particular incubation temperature did impact ethanol yields, again with similar responses among species (Fig. 5). At 12 h incubation, the ethanol concentrations increased significantly with each 10 °C rise in temperature, but after 14 days, there was no longer a difference between roots incubated at 15 and 25 °C, and they produced about twice the ethanol of roots incubated at 5 and 35 °C, which were also no longer different (Fig. 5). Complete depletion of the in situ carbohydrate pool is one explanation for identical ethanol yields from the root segments incubated at 15 and 25 °C. Had whole seedlings been tested instead of root segments, then the declining root carbohydrate pool might have been replenished from stores in the stem or foliage, thus allowing the 15 and 25 °C treatments to maintain concentration differences similar to those expressed at 12 h. It is uncertain whether species differences would have been observed at 14 days using whole seedlings.

Root responses to 35 °C over an extended period were different from responses to the other three temperatures (Fig. 5). Though they synthesized the highest ethanol for all species during the first 12 h, this faster rate was maintain only for a relatively short period, as by 24 h, three species had begun to

experience a decline (Figs. 2A–2E). After 14 days, the roots incubated at 35 °C produced about the same amount of ethanol as the roots incubated at 5 °C. End product inhibition of enzymes can be ruled out as a causal factor for this decline given the higher quantities of ethanol produced by the 15 and 25 °C treatments. A more likely cause is heat damage or impairment of one or more enzymes controlling metabolism. This is further supported by results from a preliminary experiment in which roots of Port Orford cedar were incubated at 25 and 45 °C in the same manner as the main experiment and sampled periodically from 2 to 240 h. After 2 h, both treatments produced the same amounts of ethanol. However, at the end of 240 h, the yields from roots incubated at 45 °C remained unchanged, whereas those roots incubated at 25 °C had increased by 25 times. Given that the roots were acclimated to low soil temperatures prior to this experiment, the sensitivity of their enzymes to 35 °C is not surprising.

Although ethanol synthesis maybe essential for cells and tissues to survive anoxia (Roberts et al. 1984; Bailey-Serres and Voesenek 2008), the quantity of ethanol produced over time is not always a reliable indicator of a species tolerance to this stress (Crawford and Baines 1977; Tadege et al. 1998), as there are many contributing factors influencing the ethanol produced and species can vary in their level of postanoxic tissue injury when O_2 supplies return to normal (Crawford and Braendle 1996; Greenway and Gibbs 2003; Gibbs and Greenway 2003; Bailey-Serres and Voesenek 2008). Given these limitations, it is interesting to note that anaerobic tolerance is one of many characteristics rated in the USDA NRCS (2009) PLANTS Database for the five cedars in this study. This information has been obtained from the scientific and grey literature in conjunction with field observations and is best considered an estimate. In the PLANTS Database, Atlantic white cedar is identified as having high anaerobic tolerance, yellow cedar is identified as having low tolerance, and Port Orford cedar, western redcedar, and incense cedar are all listed as having no tolerance. If 12 h ethanol synthesis alone is used as an indicator of anaerobic tolerance, then the species ranking would change somewhat, with yellow cedar being elevated to a high tolerance similar to that of Atlantic white cedar and the other three species being ranked as having low, or possibly no, tolerance.

Alternatively, rating the species anaerobic tolerance based on 14 day anoxic ethanol synthesis (Fig. 3) would still identify Atlantic white cedar as having high tolerance, followed by yellow cedar, western redcedar, and Port Orford cedar as having low to moderate tolerance, and incense cedar as having possibly no tolerance. Of the three different ratings discussed above, this last one seems more closely aligned to the species associations with wet, mesic habitats and their probability of experiencing anaerobic stress from flooding. Atlantic white cedar often grows in swampy or very wet soils (Table 1) for part of the year and clearly had the highest rates of ethanol synthesis after short or extended periods of anoxia (Fig. 3) at all temperatures (Fig. 4). Incense cedar, a species with roots that are least likely to experience flooding, more often growing on well drained, drier, hotter sites than western redcedar (Minore 1979), expressed the lowest ethanol synthesis. It is more difficult to compare environments of the three species with nearly equal, but intermediate, ethanol yields at 14 days (Fig. 3), but all three are associated with wet environments. The importance of nearby water for Port Orford cedar survival during summer may make it the species most consistently associated with wet habitats across its range (Zobel et al. 1985). Yellow cedar and western redcedar often grow together along the periphery of bogs or swamp-like sites with high water tables, especially in the southern portion of Southeast Alaska (Neiland 1971; Minore 1983; D'Amore et al. 2009), but across their ranges, these two species also thrive on deeper, more well-drained sites (Harris 1990; D'Amore et al. 2009). Therefore, some level of anaerobic tolerance might be expected for these three species. However, the higher 12 h ethanol yields for yellow cedar suggest that its roots may be better adapted to tolerate shorter periods of anoxia than Port Orford cedar and western redcedar roots. To further evaluate and rank the anaerobic tolerance of these cedars, direct comparisons of flooded whole seedlings with and without hypoxic pretreatment is needed. Checking the influence of hypoxic pretreatment is important because roots may experience hypoxia even when external O_2 supplies are not excessively limited, and any previous low O_2 exposure can make them more tolerant of anoxic stress (Johnson et al. 1989; Drew 1997). Using whole seedlings would insure that translocation of carbohydrates is taken into account in addition to the effects of postanoxic injury. Seasonal changes in species tolerance to flooding is another variable to assess (Crawford 2003).

The first objective of this project was to determine whether the production of ethanol by Port Orford cedar roots in response to anoxic shock, as would occur with rapid flooding, might contribute to its *P. lateralis* vulnerability. Given that its ethanol yields at all temperatures and incubation periods fell within the ranges produced by the other four cedar species resistant to *P. lateralis*, it is highly unlikely that this is an important factor in its greater vulnerability to this pathogen. However, it does not exclude the possibility that Port Orford cedar roots exude other constituents that alone, or in combination with ethanol, influence host recognition by *P. lateralis* zoospores.

The other objective was to determine to what extent the root incubation temperature influenced ethanol synthesis and whether the species responded similarly at different temperatures. As expected, temperature did have a major impact on the rates and amounts of ethanol produced, but in general, all species responded similarly. There were some differences in their abilities to tolerate 35 °C during the first 24 h of incubation, but they were minor. Finally, ethanol synthesis alone is limited in its ability to predict a species tolerance to anoxia. However, because literature ratings were available for all of the species that we tested, they were compared with ratings based solely on the 12 h or 14 days ethanol yields. Of these three ratings, the one based on 14 days ethanol concentrations appeared to correspond more closely to the cedars association with wet, mesic environments and their likelihood of experiencing anoxia via flooding.

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