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ETHANOL ACCUMULATION IN DROUGHT-STRESSED CONIFER SEEDLINGS

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In this study, we investigated the effect of drought stress on ethanol production and accumulation in tissues from seedlings of three conifers (Douglas-fir, lodgepole pine, and ponderosa pine) with increasing degrees of tolerance to drought stress, respectively. Significant ethanol accumulation was only observed in their aerial tissues when severely stressed (water potential < -3.0 MPa or water content < 0.4 g g^{-1} dry mass), with needles accumulating greater quantities than the sapwood or phloem. All species had the same predawn water potentials when sampled, but they were experiencing different levels of stress based on their foliar water contents. Severely stressed Douglas-fir contained higher ethanol and lower water contents (except phloem) than the more drought-tolerant ponderosa pine seedlings. Lodgepole pine with intermediate drought tolerance tended to have intermediate quantities of both components. The mechanistic basis of ethanol accumulation associated with drought stress remains to be determined, especially in larger trees. We speculate that increased levels of in situ ethanol synthesis in seedlings may be associated with heat injury (e.g., membrane damage) due to a reduction in transpirational cooling after stomatal closure. Drought-induced hypoxia may also contribute to ethanol accumulation in the sapwood.

Keywords: Douglas-fir, fermentation, lodgepole pine, ponderosa pine, water stress.

Introduction

Water stress is often a prerequisite to outbreaks of phytophagous insects (Mattson and Haack 1987; Waring and Cobb 1992; Clancy et al. 1995) and pathogens (Houston 1987; Desprez-Loustau et al. 2006) in forest ecosystems. Water deficits can weaken chemical defenses of conifers by causing decreases in production and flow of terpene oleoresins (Waring and Cobb 1992; Kolb et al. 1998) and increases in the production of stress-induced volatiles (Kimmerer and Kozlowski 1982), such as ethanol, which functions as a kairomone, attracting ambrosia and some species of bark beetles (Moeck 1970; Klimetzek et al. 1986; Liu and McLean 1989; Kelsey and Joseph 2001). In addition, if ethanol is released in binary or tertiary combinations with host monoterpenes or pheromones, then the attraction of some bark beetles, wood borers, or root weevils can be greatly increased or synergized (Tilles et al. 1986; Phillips 1988; Schroeder and Lindelöw 1989; Rieske and Raffa 1991; Byers 1992; Joseph et al. 2001; Miller 2006). For example, heavy bark beetle attack (107 beetles m^{-2}) has been recorded on severely water-stressed Douglas-fir branches with high amounts of ethanol, whereas adjacent branches with low ethanol were not attacked (Kelsey and Joseph 2001). These same bark beetles have been observed attacking drought-stressed Douglas-fir in the forest (Stoszek 1973).

Ethanol synthesis occurs whenever some form of stress impairs aerobic respiration, either by limiting the O_2 supply or by damaging the mitochondria (Drew 1997; Tadege et al. 1999). The physiological role of ethanol fermentation is the regeneration of NAD^+ that is needed in glycolysis for the continued production of pyruvate and ATP energy. This energy is critical for stabilizing cellular pH and avoiding lethal acidosis until aerobic respiration resumes, otherwise the cells die (Roberts et al. 1984, 1985; Fox et al. 1995; Gout et al. 2001; Felle 2005). Fermentation can also produce metabolic intermediates, but this may be more important in situations such as developing and germinating pollen where it occurs under aerobic conditions simultaneously with respiration (Tadege and Kuhlemeier 1997; Mellema et al. 2002). The control of ethanol biosynthesis has been hypothesized to involve cellular pH regulating the activities of the key enzymes, with activation of pyruvate decarboxylase (PDC) initiating ethanol synthesis when cellular acidity increases in response to stress (pH-stat; Roberts et al. 1984, 1985; Fox et al. 1995; Gout et al. 2001; Felle 2005). An alternative hypothesis proposes that ethanol biosynthesis is regulated by the concentration of pyruvate and enzyme kinetics (PDH/PDC-stat; Tadege et al. 1999). Under aerobic conditions, pyruvate dehydrogenase (PDH) reacts with pyruvate for use in respiration, leaving minimal amounts available for ethanol synthesis. Upon impairment of respiration, pyruvate levels will increase until sufficient amounts of PDC are reached to initiate ethanol production.

In trees, a variety of stresses can cause the tissues to produce ethanol, including flooding, pathogens, freezing, crushing, heat, pollutants, and water deficits (Kimmerer and Kozlowski 1982; MacDonald and Kimmerer 1989, 1991; Kelsey and Joseph 1998, 2003). Severely water-stressed birch (*Betula papyrifera* Marsh.) leaves at -2.5 MPa contained elevated quantities of ethanol, whereas none was detected in leaves with a potential of -1.3 MPa (Kimmerer and Kozlowski 1982). The drier leaves

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were wilted and slightly necrotic. Ebel et al. (1995) found that yellowing leaves from severely water-stressed (below −2.5 MPa) apple (*Malus domestica* Borhk.) seedlings released about half the quantity of ethanol emitted by leaves from well-watered seedlings, although the difference was not statistically significant. In this case, stomatal closure could have limited ethanol release from the stressed plants. Kelsey and Joseph (2001) induced cavitation and water stress in branches of Douglas-fir by freezing them with dry ice. After 3 wk, when water contents had declined by 60%, their ethanol concentrations were ca. 1000 and 100 times higher in the sapwood and needles, respectively, compared with controls. Aside from these observations, there is little known about the production, accumulation, or release of ethanol from drought-stressed trees.

The objectives of this study were (1) to determine to what extent ethanol accumulates in root, stem, and needle tissues of drought-stressed conifer seedlings; (2) to determine whether ethanol accumulation differs among conifer species of varying drought tolerance; (3) to determine how drought stress affects the ability of phloem tissues to synthesize ethanol; and (4) to determine whether stomatal closure and reduced photosynthesis from water stress affects carbohydrate contents of the phloem sufficiently to influence ethanol synthesis.

Material and Methods

Seedling Material

Two-year-old bare-root seedlings of ponderosa pine (*Pinus ponderosa* Dougl. ex Laws, zone 14), lodgepole pine (*Pinus contorta* Dougl. ex Loud. var. *latifolia*, zone 2), and interior Douglas-fir (*Pseudotsuga menziesii* [Mirb.] Franco var. *glauca*) were obtained from commercial sources, planted in 2-L pots in February or March 2003, and grown under ambient field conditions at the Oregon State University Botany Farm (Corvallis, OR). The potting mixture consisted of a 1 : 1 : 1 : 1 (v/v) mix of loam, peat, pumice, and fine Douglas-fir bark. Seedlings were fertilized in June 2003 with ca. 15 g of Osmocote 18 and 15 g of Scotts STEP Hi Mag Formula (Teufel Nursery, Portland, OR). Beginning on June 26, 2003, differential water treatments were applied to 20 seedlings of each species in a randomized complete block design. Water treatments were applied by two pressure-compensated drip emitters per pot designed to deliver a total of 2 L (high), 0.5 L (low), or 0 L (none) of water daily.

Sample Collection

The onset of drought stress was monitored by periodic measurements of predawn (0400 to 0600 hours) plant-water potentials on the treated seedling by excising a current-year lateral branch ($n \geq 16$) using a Scholander pressure bomb (PMS Instruments, Corvallis, OR). On the basis of these measurements, the ponderosa and lodgepole seedlings were sampled for ethanol, water content, and carbohydrates on July 22, 2003, and the Douglas-fir seedlings were sampled on August 4, 2003. On each sample date, the stem, root, and needle samples were removed from each seedling and stored on ice while being processed. Samples of 10 current-year needles from the uppermost whorl were used for constitutive ethanol analysis. A 5.0-cm stem segment was removed from 4 to 9 cm above the soil and cut into five 1.0-cm pieces. The phloem, with thin outer bark attached on the outside and probably the cambium on the inside, was removed from four pieces; three were combined in a loosely capped vial and held on ice for measurement of anaerobic ethanol production rates and one for carbohydrate analysis was air-dried in an open vial. Phloem and sapwood from the fifth stem piece were separated and analyzed for constitutive ethanol. A random sample of ca. 1.5 g of live roots was washed in deionized water, blotted dry with paper towels, and used for constitutive ethanol analysis. All tissue samples, except the phloem samples for carbohydrate analysis or anaerobic treatment, were sealed in 4-mL glass vials with screw caps lined with Teflon (polytetrafluoroethylene; PTFE) and were immediately frozen with dry ice for storage at −36°C.

Constitutive Ethanol Analysis

In preparation for analysis, frozen samples were thawed on ice, weighed into headspace autosampler vials (22 mm × 75 mm, 22 mL volume), sealed with PTFE-lined butyl rubber septa, and heated for 30 min at 102°C to deactivate any remaining enzyme activity. Constitutive ethanol contents were analyzed by the headspace gas chromatography methods outlined by Kelsey and Manter (2004). External standard curves with three calibration levels were prepared for ethanol (100%, Apper Alcohol and Chemical, Shelbyville, KY) by diluting with deionized water. A syringe was used to deliver 5.0 μL of each solution into separate autosampler vials that were analyzed interspersed with the tissue samples. Replicates of these solutions were averaged for each calibration level in the curve.

Ethanol Production Rates in Anaerobic Phloem

All conifer tissues synthesize ethanol under anaerobic conditions, with the phloem and roots producing the greatest quantities (Kelsey et al. 1998). Enzymes for ethanol synthesis are maintained in tree tissues to accommodate brief periods of hypoxia or anoxia (Harry et al. 1988; Kimmerer and Stringer 1988). In maize root tips, the synthesis of additional fermentation enzymes occurs within 2 to 4 h after the onset of hypoxia (Sachs et al. 1980; Chang et al. 2000). In tobacco roots, the activity of PDC, the enzyme that initiates and regulates ethanol synthesis, increased by 25% after 8 h of hypoxic treatment (Tadege et al. 1998). Therefore, we used phloem ethanol production rates upon exposure to short periods of anoxia (i.e., ethanol production potential), but before the onset of carbohydrate substrate limitations, as a surrogate measure of the in situ content and activity of ethanol-related enzymes. Fresh phloem samples were sealed with septa in headspace vials (22 mL) that were purged with N_2 for 30 s and then incubated in the dark at 30°C for 2, 4, or 8 h (Kelsey et al. 1998). Immediately after incubation, the samples were heated for 30 min at 102°C to deactivate enzyme activity, and the ethanol contents were analyzed by the methods described earlier for constitutive ethanol. Ethanol production rates (g ethanol g^{-1} phloem [fresh mass] h^{-1}) were determined as the slope of the linear relationship between ethanol content and incubation time.

Carbohydrate Analysis

Air-dried phloem samples were ground with a Wiley mill (40 mesh) and stored at 4°C until extraction. Soluble carbohydrates

were extracted from samples of ca. 0.05 g with three 3-mL additions of methanol : chloroform : water (12 : 5 : 3; Dickson 1979). The aqueous layer from each extraction was combined in a new vial, followed by the addition of 1 mL H_2O and 1.4 mL chloroform for phase separation of pigments and lipids (Dickson 1979). Sugars in the aqueous layer were derivatized before gas chromatography analysis, using a procedure similar to that of Eklund and Little (1998). Briefly, 250 μg of the internal standard phenyl-β-D-glucopyranoside was added to a 1-mL aliquot of the aqueous layer and evaporated to dryness. Samples were then redissolved in 100 μL of pyridine containing 2.5 mg hydroxylamine HCL and heated for 20 min at 70°C, with occasional mixing to generate the oximes of fructose and glucose. Trimethylsilyl derivatives of the sugar oximes were formed by adding 100 μL of trimethylsilylimidazole and incubating for 20 min at 70°C. Two microliters of the derivatized samples were analyzed with a Hewlett Packard 5890 Series II gas chromatograph equipped with a flame ionization detector and Phenomenex ZB-5 column (30 m × 0.25 mm inside diameter, 0.25 μm film thickness). Helium was the carrier gas, with a column flow of 1.0 mL min^{-1} and a split flow of 10 mL min^{-1} (split ratio 1 : 10). The column temperature program started at 175°C. After an initial hold of 1 min, the temperature increased at 25°C min^{-1} to a final temperature of 325°C, which was held for 6 min. Injector and detector temperatures were 350° and 375°C, respectively. Soluble carbohydrate contents (sum of sucrose, glucose, and fructose) were quantified using a four-level standard curve for each compound, with phenyl-β-D-glucopyranoside as an internal standard and derivatized by the process described earlier.

Statistical Analysis

An ANOVA was conducted using the General Linear Model procedure of SAS, version 9.1. The experiment was arranged as a randomized complete block with 20 replications for each treatment in a species. Fisher's least significant difference test was used for comparison of response variables between species and water treatments when the F-test for main effects was significant ($P = 0.05$). To meet the equal variance assumption of ANOVA, ethanol contents were log transformed for analysis. All reported values are the least squares means and pooled standard errors, except ethanol contents, which are back-transformed least squares means (median) and pooled standard errors. Tissue ethanol production potential was determined from the slope of the linear relationship between ethanol content and anaerobic incubation time (g ethanol g^{-1} phloem [fresh mass] h^{-1}), using the line-fitting procedure in Microsoft Excel 2000.

Results

Water stress imposed by the differential drip-irrigation treatments was monitored by predawn water potential measurements. Measurements obtained from the morning of sampling (fig. 1) show significant differences among treatments (high: -0.48 ± 0.08 MPa, low: -0.85 ± 0.08 MPa, none: -3.48 ± 0.08 MPa). Although sampling was done on different days (July 22 for lodgepole and ponderosa pine, August 4 for Douglas-fir), there were no significant differences in water potentials among species.

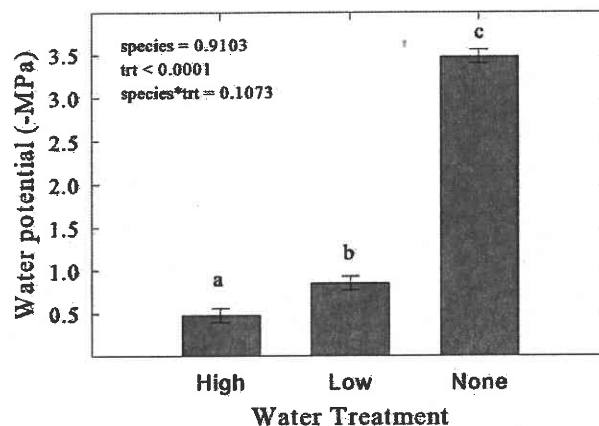

Fig. 1 Predawn xylem water potentials (MPa) in conifer seedlings exposed to various levels of drought stress at the time of sampling for ethanol, water, and carbohydrate contents. Treatments are high water (2.0 L d^{-1}), low water (0.5 L d^{-1}), and none (0.0 L d^{-1}).

Constitutive ethanol content differed among species, watering treatment, and tissues. The ethanol in needles (fig. 2*A*), sapwood (fig. 2*B*), and phloem (fig. 2*C*) increased with increasing water stress between the low- and no-water treatments for all three species. In contrast, the ethanol content in root tissues either remained unchanged (ponderosa pine) or exhibited a small but statistically significant decline with increasing water stress (Douglas-fir and lodgepole pine; fig. 2*D*). Roots were the only tissue in which ethanol levels did not show an increase with stress and the only tissues rinsed with water while sampling (to remove debris). Ethanol in flooded Douglas-fir roots can diffuse into the surrounding water (Joseph and Kelsey 1997), but whether the rinse was sufficient to reduce root ethanol contents is uncertain. Given that the root water contents in each species showed the same pattern across treatments as the other three tissues (fig. 2*E*–2*H*), we suspect the rinse had minimal effect on ethanol levels.

Within each species, constitutive ethanol contents in the various tissues were highest in the needles, followed by the phloem (except for Douglas-fir with no water), sapwood, and root tissues (fig. 2*A*–2*D*). When each treatment was averaged across all three species, the greatest change in constitutive ethanol due to drought stress (low- to no-water treatment) was seen in the sapwood (about a fivefold increase), followed by the needles (about a fourfold increase), phloem (about a twofold increase), and roots (ca. 0.7-fold change). The aerial tissues of Douglas-fir generally had the highest ethanol contents and those of ponderosa pine the least, for all treatments. In high- and low-water treatments, the ethanol in aerial tissues of lodgepole pine was intermediate between that of the other two species but usually significantly different from one or the other (fig. 2*A*–2*C*). At the most severe level of stress, ethanol in lodgepole pine sapwood and phloem was intermediate between that of the other two species (fig. 2*B*, 2*C*), whereas its needles had the highest concentrations (fig. 2*A*). Like constitutive ethanol, water content also differed among species, watering treatments, and tissues. However, the water contents of all

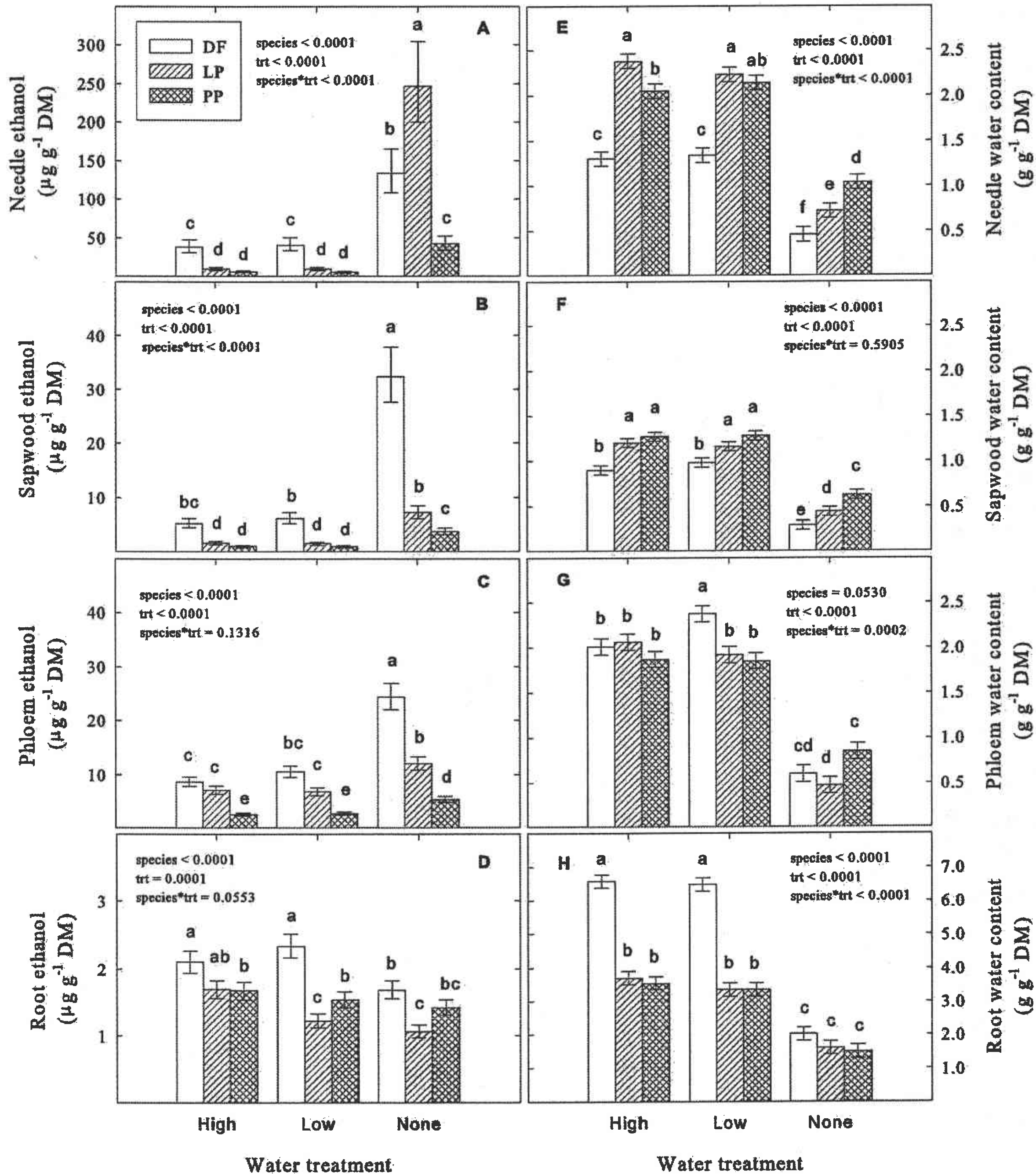

Fig. 2 Constitutive ethanol concentrations (*A–D*, back transformed) and water content (*E–H*) in tissues from conifer seedlings exposed to various levels of drought stress. DF = Douglas-fir, LP = lodgepole pine, PP = ponderosa pine. Treatments are high (2.0 L d^{-1}), low (0.5 L d^{-1}), and none (0.0 L d^{-1}). Bars with different letters are significantly different at $P < 0.05$; $n = 20$. DM = dry mass.

tissues (fig. 2*E*–2*H*) declined in each species with increasing water stress between the low- and no-water treatments.

Changes in phloem carbohydrate content in response to drought stress differed by species. They were unchanged for Douglas-fir, increased for ponderosa pine, and decreased for lodgepole pine between the low- and no-water treatments (fig. 3*A*); corresponding phloem water contents decreased between these treatments (fig. 3*B*). Phloem ethanol production potentials (fig. 3*C*), estimated by incubating tissues in anaerobic conditions, declined with increasing drought stress for Douglas-fir and lodgepole and showed no significant differences for ponderosa pine.

Phloem ethanol production potentials showed a positive log-log relationship ($R^2 = 0.800$) with tissue water content (fig. 4).

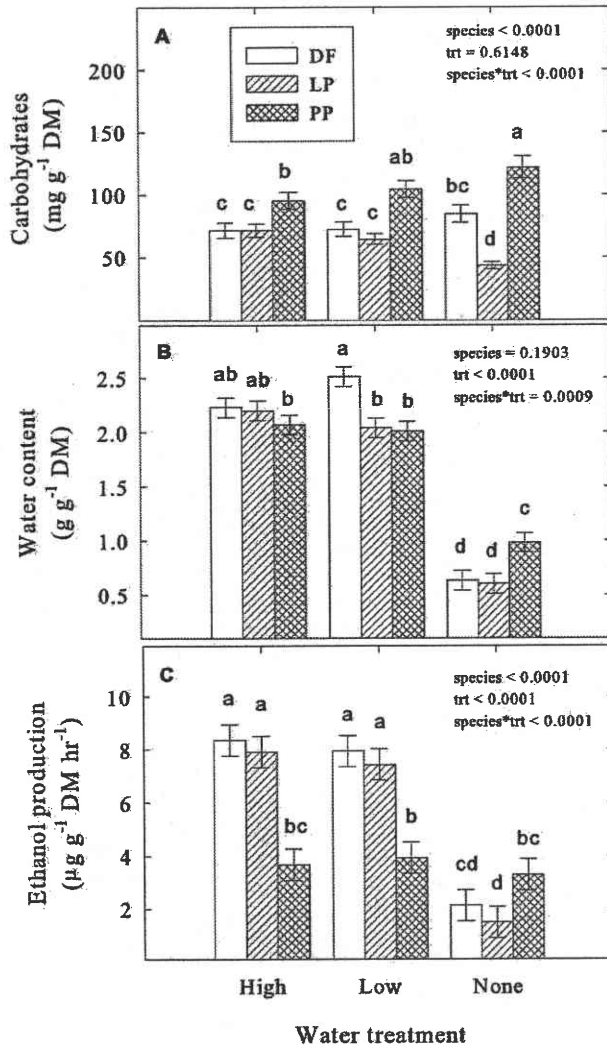

Fig. 3 Carbohydrate content (*A*), water content (*B*), and ethanol production potential (*C*) from phloem tissues of conifer seedlings exposed to various levels of drought stress. DF = Douglas-fir, LP = lodgepole pine, PP = ponderosa pine. Treatments are high (2.0 L d^{-1}), low (0.5 L d^{-1}), and none (0.0 L d^{-1}). Bars with different letters are significantly different at $P < 0.05$; $n = 20$. DM = dry mass.

In ca. 10% of the samples, the 8-h time point was not used in calculating the production potential because ethanol contents had begun to increase exponentially, indicating the induction of additional enzyme synthesis. For all Douglas-fir tissues except roots, ethanol accumulation occurred once water contents fell below ca. 0.4 g g^{-1} dry mass (fig. 5). Similar results were observed for ponderosa and lodgepole pine (data not shown).

Discussion

In this study, we examined ethanol accumulation in various tissues from seedlings of three conifer species after exposure to high-, low-, and no-water treatments. Those in the low-water treatment received 25% of the water supplied to the high-water seedlings. Although this caused a statistically significant decrease in predawn water potentials, it was not limiting enough to create a mild level of water stress (−1 to −2 MPa). The no-water treatment, however, did cause severe levels of stress (−3 to −4 MPa) similar to that reported by Anekonda et al. (2002). The proportion of severely stressed seedlings that might have recovered had they been watered at the end of our treatments was not determined. However, Pharis (1966) reports that the dry-mass water content of needles on 2-yr-old seedlings, like ours, is indicative of the whole-plant lethal point for predicting probability of mortality from water stress. Comparing the needle water contents of our seedlings (fig. 2*E*) with his values suggests that, had they been rewatered, 50%–100% of our severely stressed ponderosa pine would have lived, whereas few, if any, rewatered Douglas-fir seedlings from the same treatment group would have recovered. Pharis (1966) did not test lodgepole pine, but its hydraulic properties are similar to those of ponderosa pine (Pinol and Sala 2000). Assuming their lethal foliar water contents are similar, ca. 25%–50% of the severely stressed lodgepole seedlings might have recovered with watering. These projected levels of survival among the three species are the same as their relative ranking of drought tolerance (Minore 1979).

The amounts of ethanol accumulated varied among treatments, species, and tissue types. Within species, similar tissues from seedlings without stress in the high- and low-water treatments contained the same quantities of ethanol, except for roots of lodgepole pine (fig. 2*A*–2*D*). All aerial tissues from severely stressed seedlings contained greater ethanol contents than the corresponding tissues from seedlings without stress. When severely stressed, the least drought-tolerant Douglas-fir seedlings contained higher ethanol and lower water levels (except in phloem) than the most drought-tolerant ponderosa pine seedlings. Lodgepole pine, considered intermediate in drought tolerance, tended to have intermediate quantities of ethanol and water, with the exceptions of ethanol in the needles and water in the phloem.

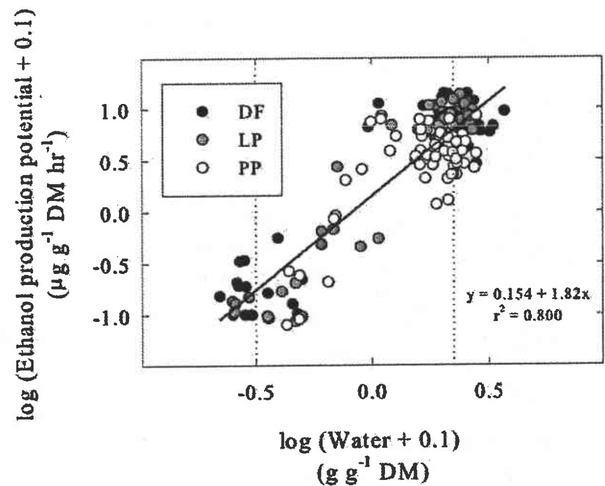

Fig. 4 Relationship between ethanol production potential and water content in phloem tissues from conifer seedlings exposed to various levels of drought stress. DF = Douglas-fir, LP = lodgepole pine, PP = ponderosa pine. Vertical reference lines correspond to water contents of 0.4 and 2.8 g g^{-1} dry mass (DM; back transformed).