Journal of Experimental Botany, Vol. 55, No. 399, pp. 1095–1103, May 2004
DOI: 10.1093/jxb/erh116 Advance Access publication March 12, 2004

Journal of Experimental Botany

RESEARCH PAPER

Ethanol synthesis and aerobic respiration in the laboratory by leader segments of Douglas-fir seedlings from winter and spring

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Received 10 June 2003; Accepted 24 January 2004

Abstract

Stem segments from terminal leaders of Douglas-fir, *Pseudotsuga menziesii* (Mirb.) Franco, seedlings were sampled in mid-December when cambial cells were dormant. The residual, debudded leaders were resampled again in early May when the cambium was metabolically active. May stems had higher constitutive ethanol concentrations than December stems. This was not the result of cambial hypoxia generated by rapid spring respiration rates, because when aerobic respiration was stimulated by incubating the stems in air at 30 °C ethanol production was induced in December, but not in May. Rapid respiration rates at 30 °C may have depleted O_2 supplies and induced ethanol production in December stems because dormant, thick-walled cambial cells may be less permeable to CO_2 and O_2, compared with metabolically active, thin-walled cambial cells in May. December stem segments incubated in a N_2 atmosphere at 30 °C synthesized 1.8 times more ethanol than segments from May, most likely because spring growth had reduced the soluble sugars available for fermentation. CO_2 efflux from May stems (after 5.5 h of incubation at 30 °C) was equal to December stems per unit volume, but greater than December stems per unit surface area. N_2-induced ethanol concentrations were positively related with CO_2 efflux per unit volume, indicating that rapidly respiring leaders can maintain rapid fermentation rates, provided soluble sugars are readily available. N_2-induced ethanol and CO_2 efflux per unit volume declined with increasing leader diameter in both seasons, whereas there were no relationships between CO_2 efflux per unit surface area and diameter. Cambium physiology and phenology influence the induction of fermentation and concentrations of ethanol produced in terminal leaders of Douglas-fir, and probably other conifers as well. This needs to be considered when comparing fermentation among species, or comparing individuals from different seasons, or disparate ages within a species.

Key words: *Pseudotsuga menziesii*, fermentation, anaerobiosis, CO_2 efflux, soluble sugars, tissue permeability, phenology.

Introduction

Low concentrations of ethanol are often present in woody tissues of healthy trees (Crawford and Baines, 1977; Kimmerer and Stringer, 1988; MacDonald and Kimmerer, 1991; Kelsey and Joseph, 1998; Kelsey *et al*., 1998*b*). When cellular O_2 supplies are plentiful, minimal quantities of ethanol are synthesized because nearly all the pyruvate produced in glycolysis is metabolized by the mitochondrial pyruvate dehydrogenase (PDH) enzyme complex and enters the tricarboxylic acid cycle for the production of ATP via oxidative phosphorylation (Tadage *et al*., 1999). However, if O_2 becomes limiting, oxidative phosphorylation stops quickly and ethanol fermentation is initiated after a brief lag period (Roberts *et al*., 1984).

Fermentation under anaerobic conditions appears to function as a back-up system to mediate cellular acidosis and generate NAD^+ so that glycolysis can produce limited

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amounts of ATP for cellular maintenance until oxidative phosphorylation is re-established (Drew, 1997). Synthesis of ethanol in the presence of O_2 (aerobic fermentation) has been shown to occur simultaneously with aerobic respiration in developing and germinating tobacco, *Nicotiana tabacum* L., pollen (Tadage and Kuhlemeier, 1997). In addition to supplying energy, aerobic fermentation in pollen may function as a shunt from pyruvate to acetyl-CoA to increase the production of important biosynthetic intermediates ((Tadage and Kuhlemeier, 1997).

Constitutive ethanol concentrations in the phloem and sapwood of healthy Douglas-fir, *Pseudotsuga menziesii* (Mirb.) Franco, in the Oregon Coast Range showed an interesting pattern over the course of a year (Kelsey and Joseph, 1998). Concentrations in both tissues were near maximum in June when spring growth was occurring, followed by a decline to low amounts during summer and early autumn. Concentrations increased in December, reached peak levels in January, and then declined again. Harry and Kimmerer (1991) postulated that the cambium in tree stems, being sandwiched between the sapwood and phloem could experience hypoxia, especially during periods of rapid growth when O_2 supplies were limited. While this could explain Douglas-fir having higher constitutive ethanol concentrations in June, it does not explain the high levels during the winter months when the cambium is dormant (Grillos and Smith, 1959; Rensing and Owens, 1994), because O_2 levels in conifer stem tissues at this time of year are similar to air (Eklund, 1990, 2000; Pruyn *et al*. 2002), and respiration rates are low since they are exclusively for tissue maintenance (Stockfors and Linder, 1998).

Constitutive ethanol concentrations in terminal leaders from Douglas-fir seedlings were expected to have higher quantities of constitutive ethanol in the spring than in the winter, because aerobic respiration rates are greater in the spring, as observed for Norway spruce (Stockfors and Linder, 1998), and this might deplete cambial O_2 supplies enough to generate greater amounts of ethanol. There was speculation that this respiration-induced fermentation could be demonstrated by incubating leader segments in air at 30 °C to accelerate the aerobic respiration rates sufficiently to induce ethanol synthesis. It was hypothesized that leader segments from May would produce greater quantities of ethanol than segments from December when induced by N_2 anoxia, because the fermentation enzymes regulating ethanol synthesis in the cambium have their highest activities in the spring, as shown for *Populus deltoides* Bartr. ex Marsh. (Kimmerer and Stringer, 1988). In addition, it was predicted that stems with higher aerobic respiration rates have the capacity to generate more ethanol than stems with lower aerobic rates when induced by N_2 anoxia.

To address these questions the terminal leaders from Douglas-fir seedlings were sampled during the winter when the cambium was dormant. The residual leaders, without their terminal buds, were then sampled again the following spring after the cambium had initiated metabolic activity. These stem segments were used to measure constitutive ethanol, and ethanol induced by incubating at 30 °C under aerobic conditions (respiration-induced ethanol) and under N_2 anoxia. CO_2 efflux was also measured for the stem segments incubated aerobically. Since there is debate over the sources and causes of variation for aerobically respired CO_2 from stems when expressed in different units (Sprugel *et al*., 1995; Levy and Jarvis, 1998; Maier *et al*., 1998; Stockfors and Linder, 1998), CO_2 efflux per unit volume and per unit surface area was calculated for comparison with ethanol production per unit dry weight.

Materials and methods

Seedlings

The 33 Douglas-fir seedlings used for this experiment became available at the completion of another study conducted at Oregon State University, Corvallis (O'Neill, 2000). Open-pollinated seeds were collected from coastal (44° 20′ N, 123° 50′ W, elevation 67–333 m) and Cascade populations (44° 50′ N, 122° 30′ W, elevation 300–833 m) in Oregon and sown into raised nursery beds containing sterilized loam in early April, 1995. Rows of seedlings were spaced 8×10 cm apart. Seedlings were fertilized, irrigated, and weeded until June 1996, and thinned in May 1997 to 50% of their original densities to minimize competition. Length and basal diameters of the current-year leaders were measured in December 1997, prior to sampling, and the diameters were measured again in May 1998.

Processing stems for constitutive and N_2-induced ethanol analysis

On 15 December 1997 the tip of each terminal leader was clipped on all seedlings, leaving 30 cm attached to the stem. The top 7 cm of each remaining leader was then sampled to measure constitutive and N_2-induced ethanol concentrations. These segments were held in plastic bags and transported immediately to a laboratory coldroom where they equilibrated to 4 °C for 30 min, and were then further processed. Needles and lateral buds were removed with sharp surgical scissors, leaving small pieces of attached petiole to minimize stem wounding. The ends of each segment damaged by the clippers (about 0.25–0.50 cm) were removed with a sharp blade and the remaining portion cut into 2 cm pieces. Two of these pieces were separately weighed into preweighed headspace autosampler vials (22×75 mm outside diameter), then sealed with a septum and crimp cap, and held on ice. The third stem piece was discarded. One vial was immediately heated to 102 °C for 30 min to deactivate enzyme activity in the stem and then analysed for constitutive ethanol concentrations (0 h of incubation at 30 °C). The other vial was used to measure N_2-induced ethanol. The headspace was flushed with N_2 gas for 30 s by piercing the septa with two syringe needles, one serving as an outlet and the other an inlet connected by tubing to a tank of compressed N_2. After incubating at 30 °C for 4 h in the dark it was heated to 102 °C for 30 min to stop fermentation and then analysed for ethanol. As described below, another segment of the leader was removed the following day for CO_2 measurements. On 30 April and 1 May 1998, the residual stems of each seedling were sampled and measured again as described here.

Ethanol analysis

Ethanol was analysed by multiple headspace extraction using an autosampler (Perkin Elmer HS40, Norwalk, Connecticut.) and gas chromatograph (Hewlett Packard 5890, Palo Alto, California, now Agilent Technologies, Wilmington, Delaware) with a J&W Scientific DB-Wax column (Folsom, California, now also Agilent Technologies). Instrument settings were as described previously (Kelsey and Joseph, 1998). After the analysis, septa were removed and the tissue dried at 102 °C for 16 h, cooled to room temperature in a desiccator box, and weighed. Ethanol concentrations were calculated from the headspace measurements (Kolb *et al*., 1984).

Processing stems for CO_2 efflux and respiration-induced ethanol analysis

Aerobic respiration was measured the next day, 16 December, with an 8 cm stem segment clipped from the tip of each remaining leader and equilibrated to 4 °C for 30 min in the coldroom before removing needles, buds, and about 0.25–0.50 cm of each end damaged by the clippers. A 4 cm portion of this segment was cut for aerobic respiration measurements and the remaining portion was processed for soluble sugar analysis as described below. Segments for respiration measurements were weighed into open vials (22×75 mm outside diameter) and held on ice until needed. A set of six segments was transferred separately into similar vials with a wet paper towel packed in the bottom to minimize drying during incubation. This set of vials was placed into a dark 30 °C incubator containing a pan of water to maintain the humidity. A similar set of samples was added to the incubator every 45 min until all replicates were processed. Aerobic respiration (described below) was measured at 5.5 h. This interval minimized any potential problem with seasonal variation in lag time between changes in stem temperature and CO_2 efflux, which can vary from 0.3 to 2 h (Stockfors and Linder, 1998). Immediately following respiration measurements, the stems were transferred to headspace vials, sealed with septa, heated at 102 °C for 30 min to deactivate enzymes, and analysed for respiration-induced ethanol generated during this treatment. After the ethanol analyses, each segment was measured for length and diameters at both ends with a digital caliper (0.01 mm). Stem volumes were calculated, assuming they were cylinders using an average diameter from both ends. Samples were then dried at 102 °C for 16 h, cooled to room temperature in a desiccator box, and weighed. These same measurements were repeated 1 May 1998, using stem segments from the residual leaders.

CO_2 efflux analysis

Aerobic respiration was measured as CO_2 efflux using a Li-Cor 6250 portable infrared gas analyser (IRGA) (Li-Cor Inc., Lincoln, NE) set up inside the 30 °C incubator. After 5.5 h of incubation (as described above) the stem segments were transferred to amber glass vials (22.7×85 mm outside diameter) sealed with PTFE silicone septa and single hole polypropylene caps. Two hypodermic needles (18 gauge), connected to the IRGA with tubing, were inserted through a vial septum. Ambient air was circulated through the vial for a few minutes to remove excess CO_2 that had accumulated in the outer phloem and bark. Not removing excess CO_2 artificially inflated efflux rates. CO_2 levels in the vial were lowered to 320–340 ppm by pumping the air through a CO_2 scrub for a few seconds, then as concentrations approached ambient (355–360 ppm) and increased linearly, the rate of CO_2 change and the rate of air flow were recorded three times. CO_2 efflux was calculated by substituting these values into the equation provided by the Li-Cor manual.

Soluble sugar analysis

Fresh stem segments for sugar analysis were heated at 102 °C in an uncapped vial for 1 h to deactivate enzymes, and then overnight for 16 h before sealing and storage at –27 °C. These dried samples were ground to 60 mesh using a Wiley mill and redried for 1 h at 102 °C prior to analysis. Soluble sugars were extracted from a 50–100 mg sample with 10 ml of deionized distilled water, as this gave better results than extraction with methanol:chloroform:water (Haissig and Dickson, 1979). Soluble sugars were determined colorimetrically (415 nm) after hydrolysis of sucrose by invertase (Blakeney and Mutton, 1980). Technical problems caused 11 samples to be dropped from the December data, giving a total n=55, rather than n=66.

Statistical analysis

All analyses were conducted with the STATGRAPHICS Plus 4.0 statistical program. Seasonal differences in ethanol concentrations, CO_2 efflux, soluble sugar concentrations, stem diameters, and the comparison of constitutive ethanol with respiration-induced ethanol concentrations were each analysed as a completely randomized design using the General Linear Model. Removal of outliers or natural log transformations of the data were used where necessary to achieve homogeneous variances and normal distributions. Transformed means and their limits defined by ±1 standard error were back-transformed for presentation. Significant differences between means were separated using Fisher's Protected LSD with model α=0.05. Simple regression analyses were used to detect relationships between variables and to identify the best fitting linear or curvilinear model based on their R^2 values.

Results

Stem characteristics and growth

All seedlings were dormant in December and had flushed in May. Terminal leaders in December had a mean length of 48.3 cm (±1.7 SE) and diameter of 0.73 cm (±0.03 SE) measured near the base. Leader diameter in May was 0.78 cm (±0.03 SE), indicating that growth had begun, but the difference was not yet significantly larger than December (P=0.194). Terminal leaders sampled in December were acclimated to mean monthly temperatures of 11.0 °C and 9.0 °C from October and November 1997, whereas the May leaders were acclimated to temperatures of 6.7 °C and 9.5 °C from March and April 1998 (Oregon Climate Service data for Hyslop station, www.ocs.orst.edu). These differences were probably not large enough to have a substantial impact on the seasonal physiological responses.

Ethanol concentrations and soluble sugars

N_2-induced (anaerobic, Fig. 1A) and respiration-induced (Fig. 1B) ethanol concentrations were 1.8 and 3.1 times higher in December than in May. By contrast, constitutive ethanol concentrations in May were 1.8 times higher than in December (Fig. 1C). Respiration-induced ethanol concentrations in December (Fig. 2A) were 3.7 times greater than constitutive concentrations, indicating that aerobic respiration at 30 °C had induced ethanol synthesis. However, in May (Fig. 2B), the constitutive ethanol was 1.8 times greater than the respiration-induced ethanol, indicating that fermentation had not been induced by aerobic respiration at 30 °C. The mean soluble sugar

concentration in leaders from December was 1.5 time higher than in May (Fig. 3).

Total and normalized CO_2 efflux

Total CO_2 efflux (μmol s^{-1}) was positively related to total stem volume (Fig. 4A) and total stem surface area (Fig. 4B). When CO_2 efflux was normalized per unit volume there was no difference between December and May (Fig. 5A). However, when calculated per unit surface area, the May CO_2 efflux was 1.2 times higher than in December (Fig. 5B).

Relationship of N_2-induced ethanol synthesis with CO_2 efflux

N_2-induced ethanol synthesis was positively related to CO_2 efflux per unit volume (Fig. 6A), showing that ethanol synthesis in December increased at a faster rate than in May, for each unit of change in CO_2 efflux. By contrast, when CO_2 efflux was expressed per unit surface area, the N_2-induced ethanol concentrations were only weakly related with the May CO_2 efflux, and there was no relationship in December (Fig. 6B).

Relationship of N_2-induced ethanol and CO_2 efflux with leader diameter

N_2-induced ethanol concentrations were negatively related to leader diameter in December and May, but both relationships were weak (Fig. 7A). Stronger negative

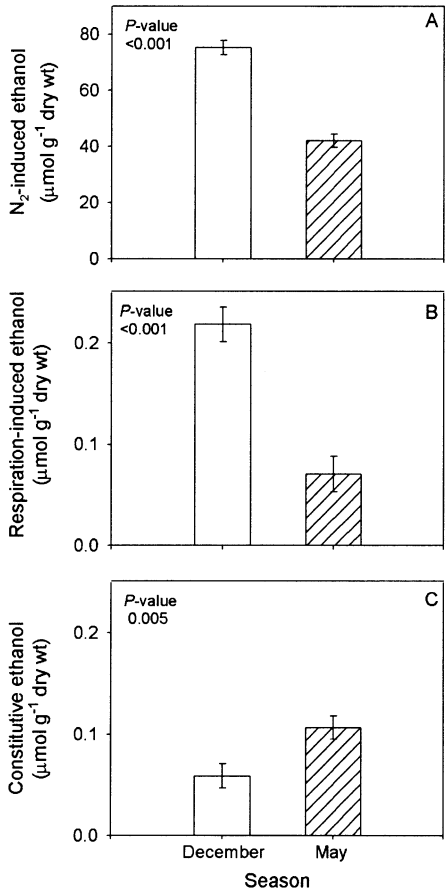

Fig. 1. Ethanol concentrations in stem segments from terminal leaders of Douglas-fir seedlings measured in December and May. N_2 -induced ethanol (A, *n*=30, 33 for December, May), respiration-induced ethanol (B, *n* = 32, 31 for December, May), and constitutive ethanol (C, *n*=30, 33 for December, May). Vertical bars are means ±SE.

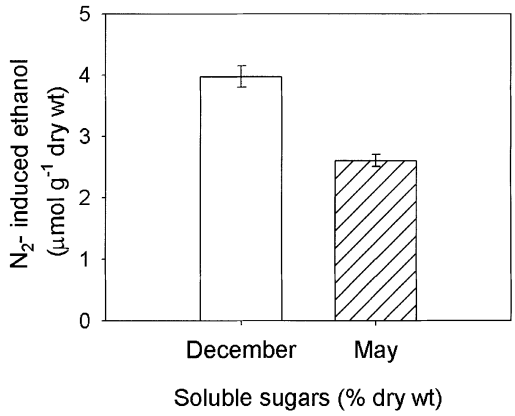

Fig. 3. Soluble sugar concentrations in stem segments from terminal leaders of Douglas-fir seedlings in December and May; *n*=23, 32 for December, May.

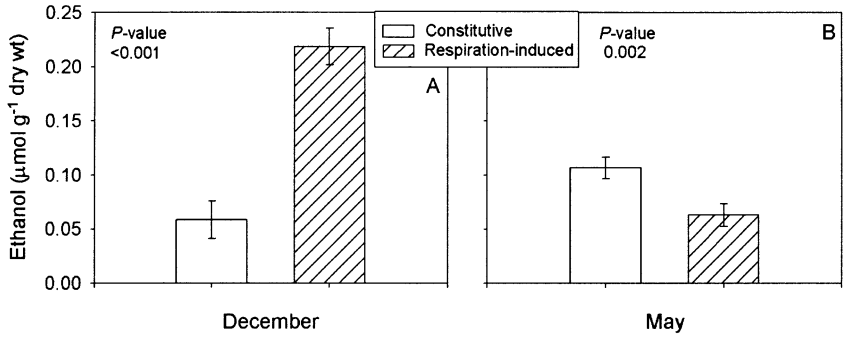

Fig. 2. Constitutive and respiration-induced (after incubating in air at 30 °C for 5.5 h) ethanol concentrations in stem segments from terminal leaders of Douglas-fir seedlings measured in December (A, *n*=30, 32 for constitutive, respiration-induced) and May (B, *n*=33, 30 for constitutive, respiration-induced). Vertical bars are means ±SE.

relationships with the leader diameter were observed for CO_2 efflux per unit volume (Fig. 7B), and there were no relationships between CO_2 efflux per unit surface area and leader diameter in either season (Fig. 7C).

Discussion

As expected, stem segments from Douglas-fir terminal leaders sampled in May contained greater amounts of constitutive ethanol than segments sampled in December. However, contrary to expectations, the December segments produced higher amounts of ethanol than those sampled in May when induced by N_2 anoxia, or when incubated in air at 30 °C for 5.5 h. Since the seedlings were grown outdoors, their phenology would be comparable to trees from other studies in the same, or similar environments (Grillos and Smith, 1959; Rensing and Owens, 1994). From these previous studies it was assumed that the cambium in the December stems was dormant because it normally lacks any anatomical changes from the end of November to the end of February, and the cambial cell walls would be at their maximum thickness.

By contrast, the cambium in leader samples from early May would be metabolically active as cell expansion and division for Douglas-fir cambium typically begins in March or April prior to bud swelling, with the most rapid division in April, May, and June (Grillos and Smith, 1959; Emmingham, 1977; Rensing and Owens, 1994). These actively dividing cambial cells and newly generated xylem and phloem cells initially have thin cell walls that thicken with age. Although cambial cells are actively dividing in early May, this represents only about 10% or less of the yearly cambial growth for Douglas-fir in western Washington (Reukema, 1965) and the Willamette Valley (Emmingham, 1977) where this experiment was conducted.

However, residual leaders sampled in May were missing their terminal bud from mid-December, and this undoubtedly had an impact on the initial cambial activity. Removal of terminal buds from *Abies balsamea* (L.) Mill. (Sundberg and Little, 1987) or *Pinus sylvestris* (L.) (Little *et al.*, 1990) shoots inhibited cambial tracheid production. For both species, replacing the bud with lanolin containing indole-3-acetic acid (IAA) promoted cambial growth. But, for Douglas-fir, some cambial cell division is still initiated in the spring after removal of the terminal bud. Bhella and Roberts (1975) clipped current year lateral branches from Douglas-fir in mid-December and stuck their bases into a rooting medium of sand and moss, then allowed them to grow for 90 d. The number of new xylem cells from cambial division was the same on cuttings with or without terminal buds, and the application of an auxin solution to the shoot base prior to planting had no effect on cambial growth. In addition, the lateral buds remaining on the residual leaders of seedlings in this experiment could help mitigate the loss of the apical bud, since they can take over the role of apical dominance when the terminal bud is

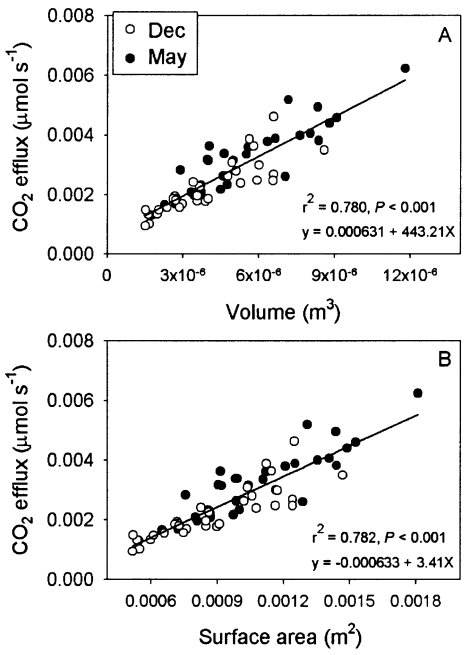

Fig. 4. Relationships of total CO_2 efflux (µmol s^{-1}) to total stem volume (A) and total surface area (B) for stem segments from terminal leaders of Douglas-fir seedlings measured in December and May; n=65 in each graph.

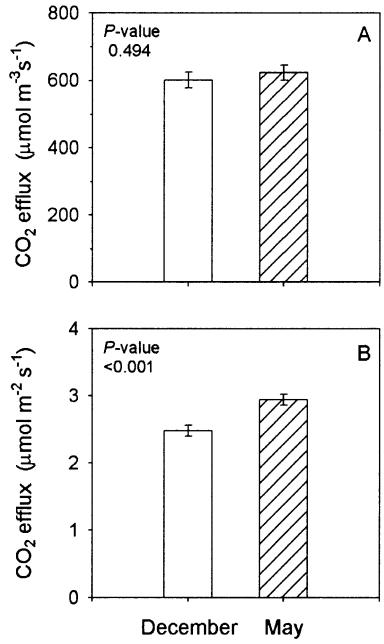

Fig. 5. Aerobic respiration by stem segments from terminal leaders of Douglas-fir seedlings in December and May expressed as CO_2 efflux per unit volume (A, n=33, 33 for December, May) and per unit surface area (B, n=33, 32 for December, May). Vertical bars are means ±SE.

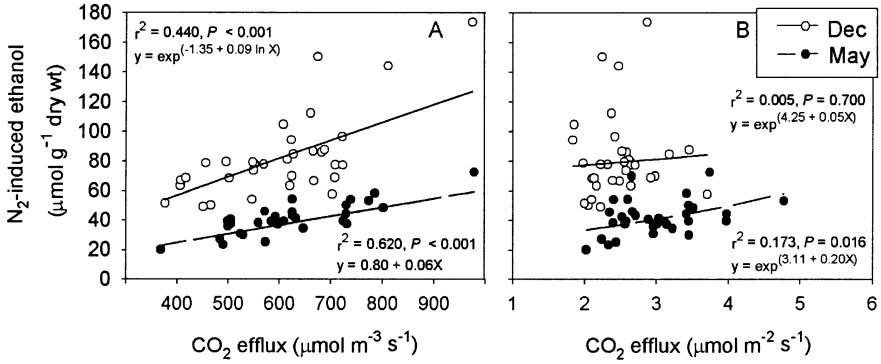

Fig. 6. Relationships of N_2 -induced ethanol concentrations to aerobic respiration expressed as CO_2 efflux per unit volume (A, n=33, 32 for December, May) and per unit surface area (B, n=33 for both months) for stem segments from terminal leaders of Douglas-fir seedlings in December and May.

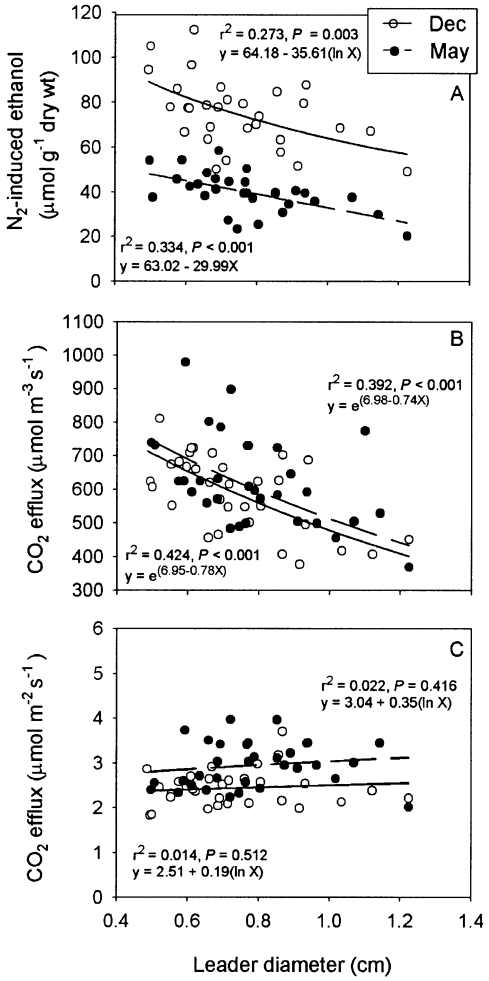

Fig. 7. Relationships of N_2 -induced ethanol concentrations (A, n=30 for both months), or CO_2 efflux per unit volume (B, n=33, 32 for December, May) and per unit surface area (C, n=32 for both months) with the terminal leader basal diameter of Douglas-fir seedlings in December and May.

removed (Frank Sorensen, PNW Research Station, personal observation during a leader clipping study, 2002). Furthermore, the terminal leaders in this experiment did exhibit a measurable increase in basal diameter between mid-December and early May, and although the difference was not statistically significant, it indicates the cambium was metabolically active. However, the level of activity was probably reduced compared with leaders with a terminal bud.

Leader segments subjected to N_2-induced anoxia produced the highest ethanol concentrations (Fig. 1A–C). But, contrary to expectations, the stems from December synthesized more ethanol than those from May (Fig. 1A). One possible explanation is that the May ethanol synthesis was limited by the lower amounts of soluble sugars (Fig. 3) as a consequence of new spring growth, which is a strong sink for carbohydrates (Kozlowski, 1992). N_2-induced ethanol synthesis in roots of Douglas-fir and ponderosa pine is related positively with soluble sugar concentrations (Kelsey *et al.*, 1998*a*), and fermentation in developing tobacco pollen is regulated by the sugar supply ((Tadage and Kuhlemeier, 1997).

Respiration-induced ethanol (Fig. 1B), resulting from stem segments being incubated in air at 30 °C for 5.5 h, exhibited the same seasonal pattern as N_2-induced fermentation (Fig. 1A), but at concentrations about two to three orders of magnitude lower. In December, the respiration-induced ethanol concentrations were 3.7 times higher than the constitutive levels (Fig. 2A), whereas the opposite was observed in May, with the constitutive ethanol concentrations 1.8 times higher (Fig. 2B). This demonstrates that rapid aerobic respiration rates induced ethanol synthesis in December, but not in May. The low amount of respiration-induced ethanol in May (0.06 μmol, Fig. 2B) was not a consequence of limited soluble sugars, since the May stems were capable of producing 42.0 μmol of ethanol when incubated for 4 h in N_2 anoxia (Fig. 1A). Furthermore, the May aerobic respiration rates were equal to, or greater than those in December (depending on their normalization Fig. 5A, B), and should have depleted internal O_2 levels at the same rate, or faster than in

December stems, but they did not, as demonstrated by their low respiration-induced ethanol concentrations (Fig. 2B).

Greater tissue permeability to gas exchange in May than in December is one possible explanation for no respiration-induced ethanol in May. Actively dividing cambium, and newly generated xylem and phloem cells in April or May have thin cell walls (Grillos and Smith, 1959; Rensing and Owens, 1994) that are likely to exchange CO_2 and O_2 more efficiently than dormant, thick-walled cells in December. Stockfors and Linder (1998) provide evidence for seasonal changes in tissue permeability for Norway spruce, *Picea abies* (L.) Karst, by recording the lag time between changes in stem temperature and subsequent changes in CO_2 evolution from the stem. The shortest lag was about 15 min in June, when stem tissues would be expected to be more permeable, and it increased in duration through the summer to a maximum of about 127 min by September (measured June to September only) as the new tissues aged. Similar seasonal changes for Douglas-fir stem permeability would be consistent with the dormant December stems producing greater quantities of respiration-induced ethanol than the metabolically active May stems.

The higher constitutive ethanol concentrations in May than in December (Fig. 1C) was expected, but apparently not the result of rapid aerobic respiration rates from the cambium inducing fermentation as demonstrated by incubating stem segments at 30 °C (Fig. 2). One possible explanation for higher May concentrations is the movement of ethanol up the stem in the transpiration stream (Joseph and Kelsey, 1997, 2000; Kreuzwieser *et al*., 1999, 2000) from sites of synthesis in new roots. Actively growing roots are likely to experience brief periods of hypoxia and ethanol synthesis (Drew, 1997). The seedlings were probably producing new roots in May, since root growth in trees typically begins about the same time as shoot growth (Larcher, 1995). In addition, spring soil temperatures in the raised beds would be warmer than the ground, thus encouraging rapid root growth and the possibility for some ethanol production. Environmental differences between the seedlings in this experiment and the 35–40-year-old Douglas-fir in the Oregon Coast Range (Kelsey and Joseph, 1998) might explain their seasonal differences in constitutive ethanol concentrations.

The N_2-induced ethanol concentrations calculated per unit dry weight were more strongly related to CO_2 efflux per unit volume (Fig. 6A), than they were to CO_2 efflux per unit surface area (Fig. 6B). This probably results from live cell weight and volume being more strongly correlated with each other than they are with the outer surface area of the stem. Periclinal division of the cambial cells (Grillos and Smith, 1959; Rensing and Owens, 1994) can add live cell volume and weight without greatly increasing the stem surface area (see photomicrographs by Rensing and Owens, 1994). Thus, if more respiring cells are added beneath each unit of surface area, the CO_2 efflux calculated on a surface area basis should be more sensitive to detecting these initial increases in live cells than calculations per unit volume. This was observed, and explains why the CO_2 efflux per unit surface area was 20% higher in May than December (Fig. 5B), compared with just a 5.3% increase for CO_2 efflux per unit volume (Fig. 5A). The positive relationship between N_2-induced ethanol concentrations and CO_2 efflux per unit volume (Fig. 6A) indicates that tissues with rapid respiration rates have the necessary constitutive proteins available (Harry *et al*., 1988) for immediate ethanol synthesis if O_2 supplies become limited.

The negative relationships of N_2-induced ethanol synthesis (Fig. 7A) and CO_2 efflux per unit volume (Fig. 7B) with leader diameter is also a function of cambial growth and differentiation. Fast- and slow-growing Douglas-fir both produce about 10 xylem cells for each new phloem cell, but faster growing trees produced a greater total number of new cells (Grillos and Smith, 1959). The majority of new xylem cells are transformed into non-living tracheids to transport water, in contrast to the cambium and phloem that remain alive near the stem surface. In Norway spruce, phloem contains 80% of the live cell volume, and most living xylem cells are concentrated in outer rings (Stockfors and Linder, 1998). The net effect is the larger diameters stems add a greater weight and volume of non-living xylem, relative to cambium and phloem, thus resulting in a dilution effect. Small diameter leaders produce more ethanol and CO_2 than larger diameter leaders because they have more metabolically active cells per unit dry weight or volume, respectively, than large leaders.

CO_2 efflux per unit surface area did not vary with leader diameter in either season (Fig. 7C) because the growth and differentiation process results in a relatively constant number of live cells per unit surface area, across a range of diameters.

In conclusion, stem segments from dormant Douglas-fir leaders in December contained lower constitutive ethanol concentrations than segments taken from the remaining debudded leaders in May, after metabolic activity in the cambium was initiated. The higher May concentrations were not caused by more rapid aerobic respiration rates associated with metabolically active cambium, because incubating the stem segments at 30 °C to stimulate aerobic respiration rates did not induced ethanol production in May, but it did in December. Seasonal differences in cambial permeability to gas exchange might be responsible for this difference. The thick cell walls of dormant cambium in December could have decreased the stems permeability to gas exchange enough that aerobic respiration rates at 30 °C were able to reduce the supply of tissue O_2 sufficiently to generate hypoxia and induce ethanol synthesis. This did not occur in the May stems because of

the greater permeability associated with the thin cell walls of metabolically active cambium. December stem segments also produced more N_2-induced ethanol than segments from May, which could be explained by the greater availability of soluble sugar substrate measured in the December stems. Interpreting the seasonal effects of stem phenology on aerobic respiration rates at 30 °C was dependent on whether CO_2 efflux was normalized by volume or by surface area. Ethanol concentrations per unit dry weight induced by N_2 anoxia were positively related with CO_2 efflux per unit volume, indicating that stems with faster respiration rates have the ability to ferment more ethanol than stems with slower respiration rates. However, these same ethanol and CO_2 efflux measurements were inversely related with stem diameters, probably because the larger leaders produce a greater proportion of non-living to living cell weight and volume, thus causing a dilution effect. The physiological and phenological status of the vascular cambium in Douglas-fir stems can influence the induction of fermentation and subsequent concentrations of ethanol produced. This needs to be taken into consideration when comparing fermentation among species, comparing individuals from different seasons, or trees with disparate ages within a species.

Acknowledgements

We thank Dr Greg O'Neill and Dr Anekonda Thimmappa for generously allowing us to use seedlings that were part of their study, Dr Connie Harrington for the use of the LiCor-6250 to make aerobic respiration measurements, Manuela Huso for statistical advice, Dr Mike Ryan and Mark Nay for reviewing an early version of this manuscript. The use of trade names is for the information and convenience of the reader and does not constitute official endorsement or approval by the US Department of Agriculture.

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