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Alcohol Dehydrogenase and Ethanol in the Stems of Trees[1]

EVIDENCE FOR ANAEROBIC METABOLISM IN THE VASCULAR CAMBIUM

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ABSTRACT

Anaerobic fermentation in plants is usually thought to be a transient phenomenon, brought about by environmental limitations to oxygen availability, or by structural constraints to oxygen transport. The vascular cambium of trees is separated from the air by the outer bark and secondary phloem, and we hypothesized that the cambium may experience sufficient hypoxia to induce anaerobic fermentation. We found high alcohol dehydrogenase activity in the cambium of several tree species. Mean activity of alcohol dehydrogenase in *Populus deltoides* was 165 micromoles NADH oxidized per minute per gram fresh weight in May. Pyruvate decarboxylase activity was also present in the cambium of *P. deltoides*, with mean activity of 26 micromoles NADH oxidized per minute per gram fresh weight in May. Lactate dehydrogenase activity was not present in any tree species we examined. Contrary to our expectation, alcohol dehydrogenase activity was inversely related to bark thickness in *Acer saccharum* and unrelated to bark thickness in two *Populus* species. Bark thickness may be less important in limiting oxygen availability to the cambium than is oxygen consumption by rapidly respiring phloem and cambium in actively growing trees. Ethanol was present in the vascular cambium of all species examined, with mean concentrations of 35 to 143 nanomoles per gram fresh weight, depending on species. Ethanol was also present in xylem sap and may have been released from the cambium into the transpiration stream. The presence in the cambium of the enzymes necessary for fermentation as well as the products of fermentation is evidence that respiration in the vascular cambium of trees may be oxygen-limited, but other biosynthetic origins of ethanol have not been ruled out.

Anaerobic metabolism in higher plants is generally considered to be a transient phenomenon, occurring when environmental or structural constraints limit the supply of O_2 to respiring tissues. Environmental constraints, particularly flooding and soil compaction, reduce the external O_2 tension leading to hypoxia in root systems. Structural constraints, particularly low surface to volume ratio and impermeable tissues, may limit the rate of O_2 movement to respiring tissues. Tissues with high rates of respiration may be particularly susceptible to O_2 deprivation. In seeds, particularly of large-seeded species such as soybean, the testa, endosperm, and cotyledons represent a significant barrier to O_2 diffusion, while embryo respiration during germination consumes O_2 rapidly. This results in an anaerobic phase during germination (17). Fruits also may produce ethanol as a result of high respiration rates during development and of a low surface to volume ratio (2).

Plant tissues which experience anaerobic conditions characteristically contain ADH[3] and PDC and are capable of ethanol synthesis (6, 8, 9, 13, 17). ADH is induced by anaerobic treatment of seeds, seedlings, or roots of a number of plant species (8, 13, 19). In maize, a coordinated induction of ADH, PDC, and a number of other glycolytic enzymes occurs in response to anaerobic induction (9, 19). The ability of plants to produce ethanol under hypoxic or anoxic conditions appears to be of adaptive significance (18), although avoidance of reduced O_2 availability (*e.g.* by the development of aerenchyma or lenticels) is certainly one of the major adaptations to anaerobic stress in plants (5, 11, 12).

These considerations led us to speculate that tissues in the stems of trees, particularly the vascular cambium, might have a low enough O_2 tension to cause normal aerobic respiration to be wholly or partially inhibited, resulting in production of ethanol or other glycolytic end products. The cambium has high respiration rates (7, 21) and is separated from the atmosphere by the secondary phloem (or inner bark) and the outer bark. Phloem consumes O_2 in respiration, and the outer bark may restrict gas exchange. O_2 tension has apparently not been measured in the vascular cambium. Chase (3) found that O_2 concentrations in the sapwood of *Populus* were about 50% of the atmospheric concentration, but the methods were somewhat crude. Hook and Brown (10) found that mass flow of air across the cambium of some woody species was restricted but did not measure diffusion rates. Tripepi and Mitchell (20) sealed stems of flooded *Acer rubrum* and *Betula nigra* seedlings with lanolin and Parafilm without affecting growth and survival, suggesting that the stems were able to tolerate anoxia. While there is no published evidence for low O_2 tensions or anaerobic respiration in the vascular cambium, preliminary evidence in our laboratory suggested that ethanol biosynthesis might be occurring in tree stems. We had found that ethanol was a ubiquitous component of the phloem and sapwood of many tree species, and that there was considerable ADH activity in the cambium (TW Kimmerer, JP Dunn, unpublished data).

In this paper, we present results from studies of ADH and PDC activity and ethanol content of the vascular cambium of several woody species, based on the hypothesis that the presence of these enzymes and of their substrates would be good indicators of fermentation. We also tested for the presence of LDH, which

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[3] Abbreviations: ADH, alcohol dehydrogenase (EC 1.1.1.1); LDH, lactate dehydrogenase (EC 1.1.1.27); PDC, pyruvate decarboxylase (EC 4.1.1.1); EtSH, 2-mercaptoethanol; pO_2, partial pressure of oxygen.

is also commonly associated with anaerobic respiration (6). We hypothesized that trees with thick bark would have greater ADH activity and ethanol content than thinner-barked trees of the same species, and we tested this hypothesis in *Acer saccharum* and two *Populus* species in forest stands at Cranberry Lake, NY. We measured seasonal trends in ADH activity in stem, roots, and leaves in *Populus deltoides* in Kentucky to determine whether there was a seasonal pattern to ADH activity similar to the seasonal pattern in cambial activity and whether ADH activity in various organs was correlated. We also examined the possibility that cambially produced ethanol and acetaldehyde were released into the transpiration stream by measuring the ethanol and acetaldehyde content of the xylem sap in transpiring and nontranspiring *P. deltoides* ramets.

MATERIALS AND METHODS

Plant Material. Ramets of *Populus deltoides* Bartr. ex Marsh. clones K417 and K403 were grown in the greenhouse as previously described (15). Ramets of clones Alton 1 and 93 were growing in a plantation in Lexington, KY, and were 8 years old at the time of sampling. *Quercus alba* L. trees were sampled at Robinson Forest, Perry County, KY. *Acer saccharum* Marsh., *Populus grandidentata* Michx., and *Populus tremuloides* Michx. were sampled in oldfields and mixed northern hardwood stands at Cranberry Lake Biological Station, Cranberry Lake, NY. We sampled a wide array of stem diameters of these species. We also sampled a few individuals of other species at Cranberry Lake to establish whether ADH and ethanol were present. All samples at Cranberry Lake were taken between June 10 and July 25, 1987.

Tissue Sampling. During periods of rapid cambial growth, the bark is readily peeled from tree stems, and there is a close correlation between the ease of bark peeling and the rate of cambial cell division (21). To obtain cambial samples, rectangular windows were cut into the bark with a knife. The bark was pried off, exposing the cambium at the interface between the secondary xylem and secondary phloem. Tissue at the xylem and phloem surface was scraped with a razor blade into centrifuge tubes containing extraction buffer (see below) for ADH, LDH, and PDC measurement, or into cold 1 M $HClO_4$ for acetaldehyde and ethanol measurement. The tubes were kept on ice and were returned to the laboratory as rapidly as practicable, usually within 30 min. Secondary phloem and xylem were removed from *P. deltoides* stems with a hammer and chisel and were quickly chilled on ice. Xylem samples consisted of the two most recent annual increments in the sapwood. Leaves and roots were harvested and processed as previously described (13). Bark thickness (outer bark plus secondary phloem) of all trees was measured with a micrometer caliper. Stem diameters were measured at breast height except in very small trees, where diameter was measured above the root collar.

Enzyme Extraction and Assay. For trees sampled in Kentucky, we used the extraction, purification, and assay methods previously described (13). In preliminary experiments, we found that the protocol developed for leaves also yielded maximal ADH activity from the cambium. All tissues were homogenized in grinding buffer (100 mM Hepes, 2 mM $MgCl_2$, 1 mM NAD, 100 mM EtSH [pH 7.55 at 4°C]) with 1 g PolyClar AT (GAF Corporation, New York) per gram tissue. Cambial tissues were homogenized in a TenBroeck homogenizer. Leaves, roots, xylem, and phloem were ground in a mortar and pestle. Xylem and phloem were finely diced with razor blades before grinding and were ground with clean sand. Tissue homogenates were filtered through Miracloth (Calbiochem) and centrifuged at 10,000*g* for 20 min, and the supernatant was desalted with Sephadex G-25, with buffer exchange into holding buffer (100 mM Hepes, 2 mM $MgCl_2$, 1 mM DTT [pH 7.55 at 4°C]). ADH was assayed by following the oxidation of NADH, with acetaldehyde as the substrate (13). LDH was assayed by following the reduction of NAD with lactate as the substrate.

At Cranberry Lake, the primitive laboratory facilities necessitated modifications of the methods. Mercaptoethanol was omitted from the grinding buffer due to the lack of fume hoods and was replaced with 50 mM DTT. Samples were not desalted prior to assay. Temperature was not strictly controllable during centrifugation or assay. We attempted to minimize the time between extraction and assay and to keep centrifuge heads and glassware cool. In Lexington, we compared the two methods, and found that the modified method yielded about 50% of the activity obtained by the standard method.

Ethanol in the Cambium. Cambial tissues were collected into cold 1 M $HClO_4$. The $HClO_4$ extracts were homogenized and then centrifuged at 5000*g* for 20 min. The supernatant was frozen for later assay. $HClO_4$ extracts were assayed by a modification of the enzymic method of Beutler (1). The reaction mixture was optimized for our experimental system and consisted of 450 mM glycine buffer (pH 9.0, adjusted with KOH), 5 mM DTT, 1.2 mM NAD, 1.0 IU yeast aldehyde dehydrogenase in 1.12 ml total volume, including 50 μl of $HClO_4$ extract. After measuring the A_{340} of the mixture, the reaction was started by the addition of 20 IU yeast alcohol dehydrogenase. The reaction was run for 10 min at 25°C, and the ethanol concentration was determined from the difference in A_{340} before and after addition of ADH.

Acetaldehyde and Ethanol in Xylem Sap. To determine whether cambially produced fermentation products might be transpired, we measured the acetaldehyde and ethanol concentration in the xylem sap of intact, transpiring *P. deltoides* ramets of two clones and in the xylem sap of ramets defoliated 24 h prior to sampling. In addition, we measured the acetaldehyde and ethanol content of flooded and control ramets of clone K417 to determine whether root anaerobiosis raised the concentration of fermentation products in the xylem sap. Xylem sap was extracted with a Scholander pressure bomb (SoilMoisture Inc., Santa Barbara, CA). Cut stems were placed in the bomb and slowly pressurized to 1.0 MPa with compressed air. The first 25 μl of xylem sap was discarded, and the next 100 μl was collected into a chilled microcentrifuge tube. The sap was frozen in liquid N_2 and assayed later for acetaldehyde and ethanol. Ethanol was assayed as described above, except that the sap was not precipitated with $HClO_4$. There was no detectable protein in the xylem sap. The acetaldehyde content was determined from the first reaction step in the ethanol assay. To correct for possible nonspecific NAD reduction, subsamples of the xylem sap were bubbled with N_2 at 25°C to drive off acetaldehyde. These subsamples reduced no NAD after bubbling.

All reagents were obtained from Sigma or Aldrich. ADH and aldehyde dehydrogenase used for ethanol and acetaldehyde assays were repurified by affinity chromatography on 5′-AMP Sepharose (Pharmacia) to eliminate residual ethanol and NADH oxidase activity. Protein assay and other methods were as previously described (13).

RESULTS

ADH Activity in the Vascular Cambium. Bark readily separated from most trees we sampled, and the cambium was easily removed as a pulpy liquid. There was substantial ADH activity in the vascular cambium of all species of trees examined (Table I). The ADH assay used for *P. deltoides* and *Q. alba* was more sensitive than that used for the other species. *A. saccharum* cambium oxidized very rapidly, becoming slightly purple before the cambium could be placed in buffer. There was no detectable LDH activity in any tree we sampled (56 trees of 9 species).

In *Populus* species, bark thickness increased linearly with stem diameter, but there was no relationship between ADH activity

Table I. *ADH Activity in the Vascular Cambium of Several Tree Species*

Data are mean and standard error of two measurements on *n* trees of each species. Trees were sampled during cambial growth in June and July.

Species	ADH Activity		*n*	Location[a]
	Mean	SE		
	μmol NADH oxidized min^{-1} g^{-1} fresh wt			
Acer saccharum	36.0	5.6	13	C
Populus tremuloides	49.4	3.2	13	C
Populus grandidentata	21.7	2.3	13	C
Populus deltoides	111.2	3.4	8	L
Betula alleghaniensis	13.6	1.2	1	C
Fagus grandifolia	17.6	2.0	1	C
Quercus alba	38.4	2.3	5	R
Pinus strobus	32.4	3.2	1	C
Tsuga canadensis	42.8	4.5	1	C

[a] Locations were Cranberry Lake Biological Station, NY (C); Lexington, KY (L); and Robinson Forest, KY (R). At Cranberry Lake Biological Station, a modified ADH extraction and assay procedure was used which was about 50% less sensitive than the method used in Kentucky (see "Materials and Methods").

and diameter (Fig. 1). In *A. saccharum*, bark thickness increased linearly with stem diameter, while ADH activity decreased (Fig. 1).

Ethanol in the Vascular Cambium. Ethanol was present in the cambium of all species. The ethanol concentrations in the cambium of trees at Cranberry Lake were: *A. saccharum*, 123.4 ± 7.6 nmol g^{-1} fresh weight; *P. grandidentata*, 56.5 ± 6.4 nmol g^{-1} fresh weight; and *P. tremuloides*, 34.7 ± 10.0 nmol g^{-1} fresh weight. There was no significant relationship between stem diameter or bark thickness and ethanol content of the cambium. The cambial ethanol concentration was negatively correlated with ADH activity in *Populus* stems (Fig. 2) but was uncorrelated with ADH activity in *A. saccharum* stems.

Cambial Growth, ADH, and PDC in Cottonwood Stems. The bark of *P. deltoides* in our plantation was loose, indicating active cambial growth, beginning on April 7, and was fairly tight, indicating a slowing of cambial growth, on most clones by September 15. ADH activity in the cambium was highest on May 12 and declined thereafter (Table II). On September 15, bark of clone 93 was still loose, and cambial ADH activity was 96.4 ± 7.0 μmol NADH min^{-1} g^{-1} fresh weight, while clone Alton 1 had tight bark, and the ADH activity was 24.9 ± 5.0 μmol NADH oxidized min^{-1} g^{-1} fresh weight. The activity of cambial ADH calculated on the basis of protein content had a seasonal trend similar to that of activity calculated on a mass basis, reaching a maximum on May 12 of 22.2 ± 4.2 μmol NADH oxidized min^{-1} mg^{-1} protein. Phloem ADH activity also had a seasonal pattern (Table II), with maximal specific activity on May 12 of 27.5 ± 2.5 μmol NADH oxidized min^{-1} mg^{-1} protein. Sapwood ADH activity was very low (Table II), and the specific activity on May 12 was 3.2 ± 0.6 μmol NADH oxidized min^{-1} mg^{-1} protein. Foliar ADH activity followed a seasonal course similar to that of the cambium, with a maximum on May 12 just after the first leaf fully expanded (Table II). The specific activity also peaked on May 12 with a specific activity of 4.2 ± 2.3 μmol NADH oxidized min^{-1} mg^{-1} protein, reflecting the high total protein content of leaves.

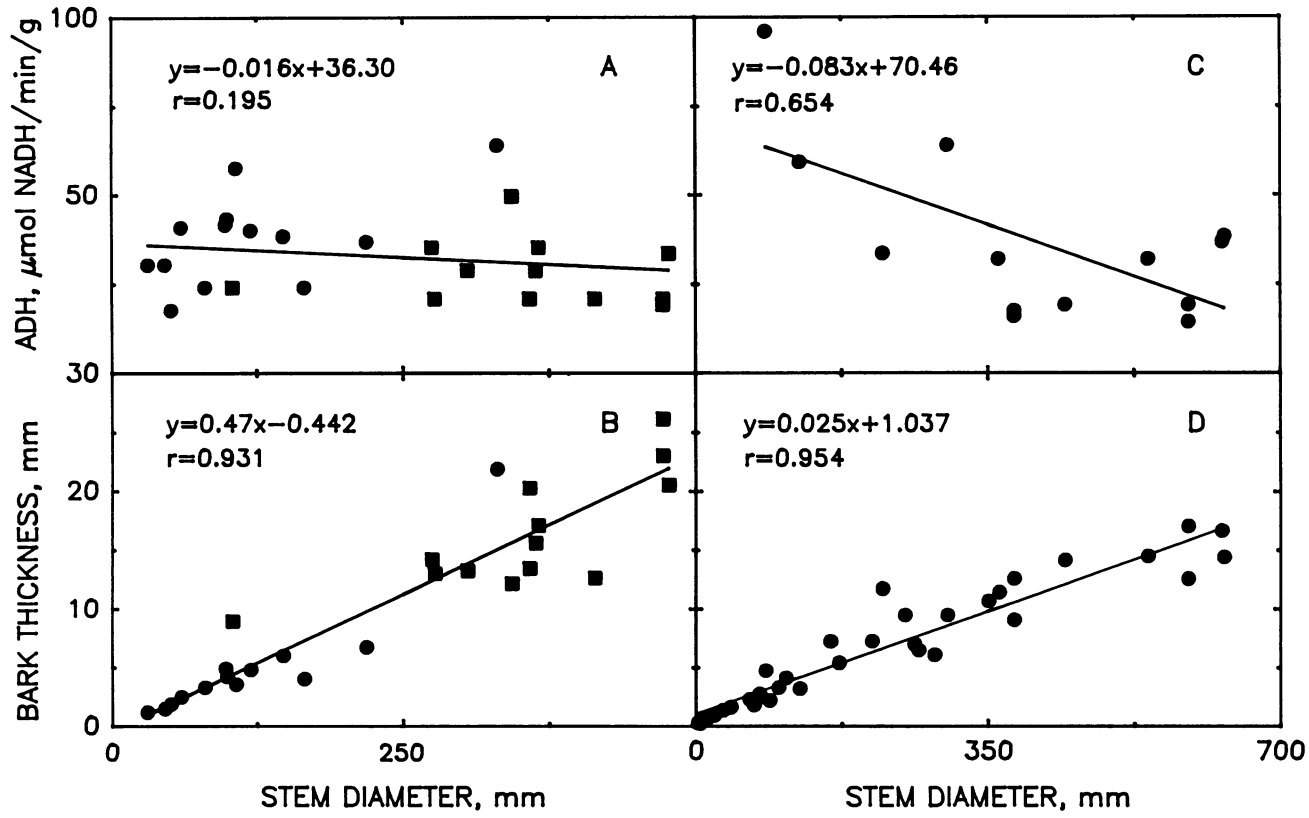

FIG. 1. ADH activity and bark thickness in *Populus* species and *A. saccharum*. A, ADH activity, and B, bark thickness in *P. tremuloides* (●) and *P. grandidentata* (■). C, ADH activity, and D, bark thickness in *A. saccharum*.

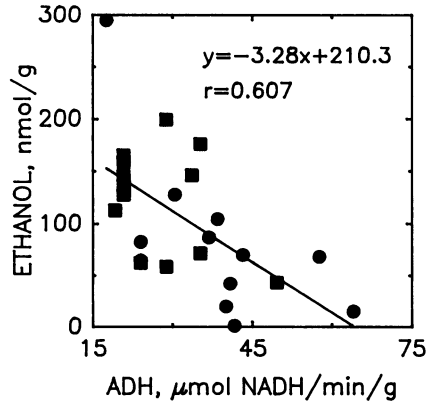

FIG. 2. Ethanol content and ADH activity in the cambium of *P. tremuloides* (●) and *P. grandidentata* (■).

Table II. *ADH Activity in Cottonwood Trees*

Data are mean ± SE of two measurements on two trees (clone 93) harvested at each date.

Tissue	ADH Activity			
	April 15	May 12	August 15	September 15
	μmol NADH oxidized min^{-1} g^{-1} fresh wt			
Leaves	NP[a]	27.6 ± 6.7	12.7 ± 4.2	0
Fine roots	12.4 ± 3.2	10.6 ± 4.2	2.3 ± 1.6	1.4 ± 0.6
Cambium	57.6 ± 4.1	164.8 ± 3.2	110.4 ± 4.3	71.6 ± 9.6
Phloem		93.2 ± 6.4	56.8 ± 3.2	8.4 ± 1.6
Sapwood		5.0 ± 1.0	2.4 ± 0.5	0.8 ± 0.8

[a] Expanded leaves not present. Phloem and sapwood samples were not taken on April 15.

Table III. *Ethanol and Acetaldehyde in the Xylem Sap of Populus Deltoides*

Data are mean and standard error of measurements on 5 trees per clone and treatment. Plants were 4-month-old greenhouse-grown ramets. Plants were defoliated 24 h before collection of xylem sap.

Clone	Control		Defoliated	
	Ethanol	Acetaldehyde	Ethanol	Acetaldehyde
	μM			
403	65.1 ± 19.2	104.1 ± 35.3	515.9 ± 78.7	317.0 ± 10.78
417	233.4 ± 23.1	153.8 ± 40.3	282.3 ± 28.4	142.6 ±26.3

We also measured PDC activity in the cambium of cottonwood. PDC activity on May 12 was 26.4 ± 6.4 μmol NADH oxidized min^{-1} g^{-1} fresh weight, with a specific activity of 3.5 ± 1.4 μmol NADH oxidized mg^{-1} protein. On September 15, PDC activity was 10.2 ± 4.1 μmol NADH oxidized min^{-1} g^{-1} fresh weight, with a specific activity of 1.4 ± 0.8 μmol NADH oxidized min^{-1} mg^{-1} protein.

Ethanol in the Transpiration Stream of Cottonwood. Acetaldehyde and ethanol were present in the transpiration stream of 4-month-old ramets of two clones of *P. deltoides* (Table III). The ethanol content of foliated ramets of clone K403 was lower than that of clone K417 ramets. The leaf area of clone 403 ramets was considerably greater than the leaf area of clone K417 ramets. Defoliation of clone K403, which had rapid cambial growth, resulted in an eightfold increase in xylem sap ethanol, and a threefold increase in xylem sap acetaldehyde (Table III). There was no significant increase in xylem sap acetaldehyde or ethanol when ramets of clone K417 were defoliated. Cambial growth was slower in clone K417 than in K403, as indicated by tight bark and a dry cambial surface. Flooding of roots did not increase xylem sap ethanol or acetaldehyde concentrations: when clone K417 plants were flooded with N_2-purged nutrient solution for 3 d, xylem sap acetaldehyde and ethanol concentrations did not change significantly. We did not examine the response of clone K403 plants to flooding.

DISCUSSION

ADH and ethanol were present in the cambium of all trees examined, and PDC was present in *P. deltoides* cambium. These enzymes are also present in tree leaves (13, 15), but leaves do not normally produce acetaldehyde or ethanol except when exposed to stress from acidic air pollutants or to experimentally imposed anaerobic stress (14, 15; RC MacDonald, TW Kimmerer, unpublished data). The presence of both of the enzymes neccesary for fermentation and of the fermentation products themselves strongly suggests that at least some respiratory metabolism in the vascular cambium is fermentive. The trees examined were apparently healthy, and while we cannot rule out the possibility of a microbial contribution to the observed fermentation, it seems unlikely that all the trees we sampled were infected. These results suggest that respiration in tree stems may be O_2 limited due both to diffusional resistance in the outer bark, phloem, and cambium and to rapid O_2 consumption by the phloem and cambium. Direct measurements of cambial pO_2 and O_2 consumption will need to be made to confirm this hypothesis.

We had anticipated that trees with thicker bark, and therefore a longer diffusion pathway for O_2, would have higher ADH activity and/or greater ethanol synthesis. This was not the case. In *A. saccharum*, the relationship between bark thickness and ADH activity was a negative one, while in *Populus* species there was no significant relationship (Fig. 1). Similarly, ethanol concentrations were unrelated to stem diameter and bark thickness. While larger trees have thicker bark (Fig. 1), they often grow and respire more slowly than do smaller trees of the same species (16, 21). Since high cambial growth rates would require high cambial respiration rates, the relationships we expected between ADH activity or ethanol content and bark thickness may be confounded by differences in cambial respiration rates.

It seems very unlikely that respiration in tree stems could be entirely anaerobic, given the low molar yield of ATP in fermentation as compared with mitochondrial respiration. If respiration rates are very high in the cambium, such that O_2 becomes limiting, it is more likely that a partitioning of carbon between aerobic and anaerobic pathways occurs. The operation of both pathways under hypoxic conditions would allow some complete oxidation of substrate, but maintain higher glycolytic rates, and higher rates of cytoplasmic NADH reoxidation. The partitioning between these pathways could be regulated by cytoplasmic pH and by competition between PDC and the pyruvate dehydrogenase complex (6, 18).

It is also possible that ethanol is synthesized in the cambium by some other pathway. Although we know of no other pathway leading to ethanol, the enormous biochemical diversity of plants makes us cautious about interpreting our results exclusively in terms of O_2 limitations on respiration. In *Populus*, there was an inverse relationship between cambial ethanol and ADH activity (Fig. 2), which could be an indication that ADH consumes, rather than produces, ethanol in the cambium. In addition to direct measurements of cambial pO_2, it will be neccesary to rule out other sources of ethanol before we can be certain that anaerobic fermentation does occur in tree stems.

Although anaerobic respiration is considered less efficient than aerobic respiration, this is not neccesarily the case if the fermentation products are fully utilized elsewhere. In mammals, lactate produced in muscle during periods of anaerobic activity is transported to the liver and oxidized fully, so that ATP yields on a whole-organism basis are the same as for aerobic respiration (4).

A similar process could occur in trees if ethanol produced by the cambium is remetabolized in the leaves. Ethanol was present in the xylem sap and increased in concentration when transpiration was inhibited by defoliation in a *P. deltoides* clone with rapid cambial growth. Flooding of roots did not increase xylem sap ethanol in a clone with slow cambial growth. These results are circumstantial evidence for a cambial (or at least stem) origin of ethanol in the transpiration stream. Ethanol in the transpiration stream will be carried to the leaves, where there are two possible fates. The ethanol may evaporate or may be metabolized in leaf mesophyll cells. In extensive experiments, we have been unable to detect ethanol evaporating from leaves of aerobic, unstressed plants (14, 15; RC MacDonald, TW Kimmerer, unpublished data). The amounts of ethanol we have observed in leaves of pollution-stressed or anaerobic leaves are several orders of magnitude greater than would be delivered in the transpiration stream (14, 15). Our methods for foliar ethanol measurement in pollution-stressed plants use headspace analysis of detached leaves and might not detect ethanol delivery in the xylem sap, so we cannot at present rule out the possibility of ethanol evaporation from leaves of transpiring plants.

ADH is present at high activity in cottonwood leaves (13; Table II), and reduction of acetaldehyde to ethanol is readily reversible. ADH in leaves may serve to metabolize ethanol delivered in the transpiration stream. This would account for the high activity of ADH in tree leaves and the lower activity or absence in herbaceous plants which lack cambial growth. It will be necessary to determine whether aldehyde dehydrogenase or other enzymes capable of utilizing acetaldehyde are present in tree leaves. The presence both of ADH and aldehyde dehydrogenase would allow synthesis of acetate from ethanol, and, on a whole-tree basis, would mitigate the respiratory inefficiency of fermentation in the cambium.

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