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Alcohol Dehydrogenase and Pyruvate Decarboxylase Activity in Leaves and Roots of Eastern Cottonwood (*Populus deltoides* Bartr.) and Soybean (*Glycine max* L.)[1]

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ABSTRACT

Pyruvate decarboxylase (PDC, EC 4.1.1.1) and alcohol dehydrogenase (ADH, EC 1.1.1.1) are responsible for the anaerobic production of acetaldehyde and ethanol in higher plants. In developing soybean embryos, ADH activity increased upon imbibition and then declined exponentially with development, and was undetectable in leaves by 30 days after imbibition. PDC was not detectable in soybean leaves. In contrast, ADH activity remained high in developing cottonwood seedlings, with no decline in activity during development. ADH activity in the first fully expanded leaf of cottonwood was 230 micromoles NADH oxidized per minute per gram dry weight, and increased with leaf age. Maximal PDC activity of cottonwood leaves was 10 micromoles NADH oxidized per minute per gram dry weight. ADH activity in cottonwood roots was induced by anaerobic stress, increasing from 58 to 205 micromoles NADH oxidized per minute per gram dry weight in intact plants in 48 hours, and from 38 to 246 micromoles NADH oxidized per minute per gram dry weight in detached roots in 48 hours. Leaf ADH activity increased by 10 to 20% on exposure to anaerobic conditions. Crude leaf enzyme extracts with high ADH activity reduced little or no NADH when other aldehydes, such as trans-2-hexenal, were provided as substrate. ADH and PDC are constitutive enzyme in cottonwood leaves, but their metabolic role is not known.

Pyruvate decarboxylase and alcohol dehydrogenase catalyze the decarboxylation of pyruvate to acetaldehyde and the reduction of acetaldehyde to ethanol, with concomitant oxidation of NADH. Despite some continuing controversy (2), production of ethanol under anaerobic conditions appears to be an important mechanism by which plants may survive and continue respiration under conditions of transient hypoxia, although few plants can survive prolonged anoxia (8).

ADH[2] is a particularly well-studied enzyme in plants, and is inducible in roots of several species upon exposure to anaerobic conditions. Upon exposure of maize roots to anaerobiosis, for example, normal protein synthesis ceased, and a characteristic set of anaerobic polypeptides appeared, among which ADH polypeptides were major products (20). This response was similar to the effect of mild heat shock, and represented a coordinate genetic response to flooding stress (6). However, when maize leaves were exposed to anaerobic shock, there was no incorporation of ^{35}S-methionine into new protein, and the characteristic anaerobic polypeptides did not appear, leading to the conclusion that the maize leaf ADH genes were totally repressed (19).

Seeds often contain substantial activities of ADH. In maize scutella, this activity declined rapidly with time after imbibition, in part because of lack of *de novo* enzyme synthesis, and in part because of accumulation of a polypeptide inhibitor of ADH, which irreversibly inactivated ADH (7, 13). Upon germination, ADH activity in maize declined to undetectable levels in the cotyledon and young leaves (12). Similarly, in soybean cotyledons (11) and in maize and pea seeds (16), ADH activity peaked shortly after imbibition and thereafter declined to undetectable levels. The peak in ADH activity during imbibition was associated with an anaerobic or hypoxic phase, due either to limiting O_2 transport or to mitochondrial immaturity (16).

PDC is a much less studied enzyme, though it probably represents the regulatory step in ethanol biosynthesis (3). PDC is present in germinating pea seeds (17) and in roots (14) but has not previously been shown to occur in mature leaves.

The behavior of ADH and PDC in most studies is consistent with the view that these proteins are absent or present only in trace amounts in mature leaves, and that the ADH genes are repressed. However, we have observed that leaves of woody plants exposed to SO_2 stress produce ethanol aerobically (9) and that leaves of a large number of plant species produce ethanol anaerobically (10). Since PDC and ADH are the only enzymes known to catalyze ethanol synthesis, this is presumptive evidence of their occurrence in leaves. The purpose of this study was to directly determine whether PDC and ADH were present in higher plant leaves, and to compare activities of ADH in leaves and roots of cottonwood (*Populus deltoides* Bartr.), whose leaves produce abundant ethanol anaerobically, and in leaves and roots of greenhouse-grown soybean (*Glycine max* L.), whose leaves do not produce significant amounts of ethanol anaerobically (10).

MATERIALS AND METHODS

Plant Material. Cottonwood cuttings (clone K417) were grown in the greenhouse as previously described (10). For developmental studies of ADH in germinating seeds and young seedlings, soybean and cottonwood seeds were surface sterilized with 5% H_2O_2 for 30 min, rinsed with distilled water, and sown on moist

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[2] Abbreviations. ADH, alcohol dehydrogenase (EC 1.1.1.1); PDC, pyruvate decarboxylase (EC 4.1.1.1); LDH, lactic dehydrogenase (EC 1.1.1.27); TPP, thiamine pyrophosphate; EtSH, mercaptoethanol; LPI, leaf plastochron index; PPFD, photosynthetic photon flux density.

sterile filter paper in Petri dishes. The Petri dishes were incubated in a growth chamber, and seedlings were transplanted into Spencer-LeMaire bookplanters (Spencer-LeMaire Industries, Edmonton, Alberta, Canada) filled with 1:1 perlite:vermiculite. Seedlings were watered daily with one-eighth strength Hoagland solution. Growth chamber environmental conditions were: 25°C d/16°C night; 70 ± 5% RH; 16 h photoperiod; PPFD 1100 ± 250 μmol m^{-2} s^{-1} at plant tops, from low pressure sodium, cool-white fluorescent and incandescent lamps.

Anaerobic Treatments. Two types of anaerobic treatments were applied: intact plants were flooded with nitrogen-purged nutrient solution, and detached organs were incubated in N_2 atmospheres. For the flooding experiments, greenhouse-grown plants were carefully removed from the pots and the roots were immersed in 100 L containers of one-eighth strength Hoagland solution. The containers were bubbled with compressed air for 24 h. At the start of the experiment, half of the containers were switched to N_2 bubbling, while in the remainder, air was bubbled. In some experiments, there was no preliminary bubbling with air prior to the start of the experiment.

For detached organ experiments, plants were removed from pots, placed in the 100 L containers and bubbled with air for 24 h. Leaves and roots were then detached and carefully placed in 500 ml separatory funnels, avoiding damage to tissue. The funnels were sealed with a septum below the stopcock, and were purged with N_2 or air via a needle inserted through the septum. The funnels were then incubated in the growth chamber or on the laboratory bench at 25°C.

Enzyme Extractions. In preliminary experiments, the enzyme isolation procedure was optimized for each plant species and organ (leaf or root), with the use of large amounts of cofactors, reductants and phenolic binding agents necessary for enzyme isolation from woody plants (see Ref. 5 for a discussion). Cottonwood leaves were ground in a mortar and pestle at 4°C in grinding buffer (100 mM HEPES, 2 mM $MgCl_2$, 1 mM NAD, 2 mM TPP, 1 g PolyClar AT [GAF Corporation, New York], 100 mM EtSH [pH 7.55 at 4°C]) at a ratio of 10 ml buffer/g fresh weight plant tissue, and the homogenate was filtered through Miracloth (Calbiochem). TPP and $MgCl_2$ were added to improve recovery of PDC. Their presence had no effect on recovery of ADH. The filtrate was centrifuged at 10,000*g* for 20 min at 4°C. The supernatant was then passed through a Sephadex G-25 column (Pharmacia) to remove low mol wt substances, with buffer exchange into holding buffer (100 mM HEPES, 2 mM $MgCl_2$, 1 mM DTT, 2 mM TPP [pH 7.55] at 4°C), and the protein fraction was collected. The preparation was kept at 1°C and assayed within 2 h. For cottonwood roots, the same procedure was used, except that the EtSH concentration in the grinding buffer was reduced to 20 mM, and the PolyClar AT was reduced to 500 mg. For isolation of ADH from soybean leaves and roots, the EtSH concentration was reduced to 10 mM, and the pH was reduced to 7.4. PolyClar AT was omitted from the grinding medium for soybean roots.

Enzyme Assays. Assay conditions were optimized for each plant species and tissue type. The preparations were assayed for ADH by following the oxidation of NADH at 340 nm. The reaction buffer for cottonwood extracts consisted of 100 mM Mes, 5 mM $MgCl_2$, 1 mM DTT (pH 6.25 at 25°C). To 2 ml reaction buffer, 250 μg NADH was added, followed by 100 μl protein extract. The A_{340} was followed for 2 min for determination of NADH oxidase activity, after which the reaction was started by addition of 100 μl 70% acetaldehyde. The A_{340} was measured for 2 to 5 min. Results were calculated as μmol NADH oxidized min^{-1} g^{-1} dry weight after correction for NADH oxidase activity. For soybean roots, the optimum pH was 6.5 at 25°C. For soybean leaves, optimization was not possible due to lack of detectable ADH activity. Cottonwood leaf preparations were also analyzed with a variety of aldehydes as substrate, including *trans*-2-hexenal, propionaldehyde, and formaldehyde. *Trans*-2-hexenal was emulsified in the buffer by sonication just prior to addition of protein.

PDC activity was measured by coupling pyruvate decarboxylation to NADH oxidation by ADH. The reaction buffer consisted of 100 mM Mes, 5 mM $MgCl_2$, 1 mM DTT, 2 mM TPP (pH 6.1 at 25°C). NADH (250 μg) was added to 2 ml of reaction buffer, followed by 100 μl protein extract and 30 units of yeast ADH. NADH oxidase activity was determined by measuring A_{340} for 2 min prior to the addition of 3.7 μg pyruvic acid. The reaction was then followed for 10 min.

Protein concentrations were measured by the method of Bradford (1), using purified spinach RuBPCase (Sigma) as standard, and BioRad Bradford reagent (BioRad). The fresh weight/dry weight conversion was determined on a subsample of each organ harvested for enzyme extraction. Sephadex was obtained from Pharmacia Fine Chemicals. All other reagents and enzymes were obtained from Sigma. Pyruvate was passed through Sephadex LH-20 to remove polymerized material just before use.

RESULTS AND DISCUSSION

ADH and PDC Activity in Soybean and Cottonwood. ADH and PDC were not detectable in leaves of 30 or 90-d-old soybeans grown in the greenhouse. When detached leaves of 30-d-old plants were incubated in anaerobic conditions for 24 h, severe necrosis occurred and there was no induction of ADH or PDC activity. Anaerobically incubated leaves of greenhouse-grown soybeans did not produce acetaldehyde or ethanol (10). Leaves of field-grown soybeans were competent to produce acetaldehyde and ethanol anaerobically (10), suggesting that under some circumstances, ADH and PDC can be induced in soybean leaves. In soybean roots, ADH activity was 36 ± 8 μmol NADH oxidized min^{-1} g^{-1} dry weight for 30-d-old plants, and 18 ± 6 μmol NADH oxidized min^{-1} g^{-1} dry weight for 90-d-old plants. Root PDC activity was 7 ± 2 μmol NADH oxidized min^{-1} g^{-1} dry weight at 30 and 90 d.

ADH and PDC activity were present in leaves of greenhouse-grown cottonwood. ADH activity in cottonwood leaves was a function of leaf age (Fig. 1). Unless otherwise stated, subsequent experiments were done with leaves LPI=6 or 7. Leaves harvested early in the morning were very low in ADH activity, suggesting a diurnal periodicity in ADH activity. Early morning samples were excluded from the data presented.

PDC activity in leaves and roots of cottonwood was lower than ADH activity, with maximal activities of 10 ± 3 μmol NADH oxidized min^{-1} g^{-1} dry weight in leaves and 8 ± 2 μmol NADH oxidized min^{-1} g^{-1} dry weight in roots. The low activity may reflect difficulties in isolation of this labile enzyme, rather than the true *in vivo* activity.

Cottonwood root ADH activity was 141 ± 20 μmol NADH oxidized min^{-1} g^{-1} dry weight (n = 4) when roots were taken

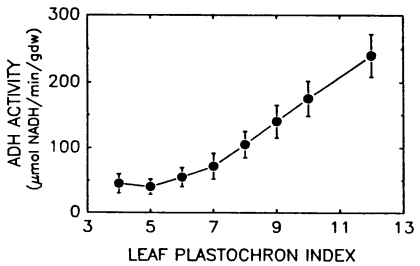

FIG. 1. ADH activity as a function of leaf age in rooted cottonwood cuttings. Leaf Plastochron Index is directly proportional to leaf age (15), with an expanding leaf 2 cm long as the index leaf (LPI=0). Plants were 3-month-old rooted cuttings of clone UK417.

directly from pots. When plants were carefully removed from pots, and the root were immersed in air-bubbled nutrient solution, root ADH activity decreased to 48 ± 16 μmol NADH oxidized min^{-1} g^{-1} dry weight ($n = 4$) within 24 h.

ADH Activity in Developing Seedlings. ADH activity is high in the seeds and embryos of many plants, and declines with development (7, 11, 13). I compared the changes in ADH activity in embryos of developing soybeans with those of cottonwood. ADH activity of soybean embryos increased significantly upon imbibition, followed within 3 d by an exponential decline (Fig. 2). ADH activity in leaves declined to very low levels within 15 d and was undetectable by 30 d. Soybean root ADH activity remained at a constant, low level from 15 d on. This pattern is consistent with other observations of changes in embryo and leaf ADH activity in a variety of plant species grown under aerobic conditions (*e.g.* Refs. 12, 16). The increase in activity following imbibition may be due to inhibited O_2 transport into the rapidly respiring embryo (16).

ADH activity in cottonwood embryos did not increase upon imbibition, and did not decline during development (Fig. 2). By 15 d after imbibition, leaf ADH activity was slightly higher than was root ADH activity.

Effects of Anaerobiosis on ADH Activity. Exposure of roots of intact cottonwood plants to N_2 resulted in substantial increases in ADH activity in roots and in small but significant increases in leaf ADH activity. When plants were transferred directly from pots to N_2-bubbled nutrient solutions, root ADH activity increased within 24 h but then decreased dramatically by 48 h (Table I). This decrease was accompanied by the appearance of grossly necrotic tissue. There was a large decrease in the root ADH activity of air-bubbled controls during the first 24 h (Table I). Evidently, root ADH was already partially induced by hypoxia in the pots, which was relieved upon bubbling with air. Roots were unable to survive the additional anaerobic stress.

When plants were first bubbled with air for 24 h, then shifted to N_2, roots did not become necrotic, and root ADH activity increased over the entire experiment (Table II). The aerobic pretreatment allowed the roots to survive 48 h of anaerobic stress.

Changes in leaf ADH activity due to root hypoxia were small (Tables I and II). Transplanting into air-bubbled nutrients had no effect on leaf ADH activity (Table I). Exposure of roots to anaerobic conditions resulted in increases in leaf ADH activity of only 10 to 20% (Table II). Increased ADH activity in leaves upon exposure of roots to anaerobic conditions suggests the translocation of an inducing signal. Acetaldehyde or ethanol produced in hypoxic roots could be translocated to leaves via the transpiration stream. A moderate increase in ADH activity was observed when maize seedlings were incubated in 50 mM acetaldehyde or ethanol (4). Incubation of pea cotyledons in acetaldehyde or ethanol prevented or delayed the normal developmental decline in ADH activity following germination (11).

Exposure of detached roots to N_2 resulted in large increases in ADH activity (Table III), similar to those observed with intact plants (Table II). Incubation of detached leaves in N_2 resulted in small (13%) increases in ADH activity (Table III). Plants used in this experiment were preincubated in air-purged nutrient medium for 24 h prior to detaching the organs. When roots were taken directly from pots and incubated in N_2-purged vessels, ADH activity declined rather than increasing, and roots were necrotic at 24 h (data not shown).

As in other plants (6, 11, 12, 16, 19, 20), ADH in cottonwood roots appears to be an inducible enzyme, whose activity increases upon exposure to hypoxia and decreases upon exposure to O_2. Activity of the root enzyme decreased when plants were moved from soil to air-bubbled nutrient solution (Table I) and increased when exposed to hypoxic nutrient solutions (Tables I–III).

ADH activity in leaves, on the other hand, appeared to be constitutive, remaining at high levels throughout development (Figs. 1 and 2) and changing only slightly with changes in O_2 availability (Tables I–III). Alternatively, leaf ADH could be

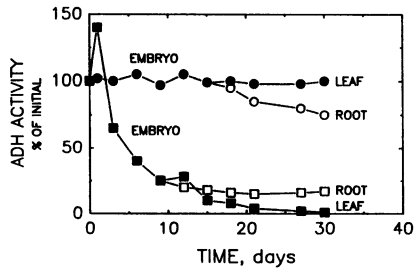

FIG. 2. ADH activity as a function of time since imbibition in cottonwood (●) and soybean (■) seedlings. ADH activity was measured on whole embryos (minus cotyledons) until there was sufficient plant mass to be able to separate leaves and roots. Initial activity of embryos (minus cotyledons) from dry seeds was 232 μmol NADH oxidized/min·g dry weight for cottonwood and 185 μmol NADH oxidized/min·g dry weight for soybean.

Table I. *ADH Activity in Leaves and Roots of Cottonwood*

Roots of intact plants were bubbled with air or N_2 beginning at time 0. At 0, 24, and 48 h, six plants in each treatment group were harvested for determination of ADH activity. Data are mean ± SE. Asterisks denote significant differences between control and treatment (t test, $P < 0.05$). Plants were 3-month-old rooted cuttings of clone UK417. Leaves with LPI = 6 and 7 were used for ADH assay.

		ADH Activity at Time		
		0	24	48 h
		μmol NADH oxidized/min^{-1}·g^{-1} dry wt		
Root	Air	180 ± 18	86 ± 12	72 ± 4
	N_2	178 ± 23	340 ± 26*	21 ± 11*
Leaf	Air	223 ± 8	236 ± 18	204 ± 11
	N_2	236 ± 12	268 ± 21*	256 ± 16*

Table II. *ADH Activity in Leaves and Roots of Intact Cottonwood*

Roots of intact plants were bubbled with air for 24 h before the start of the experiment. At time = 0, half the plants were switched to N_2 and the remainder were bubbled with air. Details as in Table I.

		ADH Activity at Time		
		0	24	48 h
		μmol NADH oxidized/min^{-1}·g^{-1} dry wt		
Root	Air	51 ± 8	56 ± 6	48 ± 4
	N_2	58 ± 4	102 ± 8*	205 ± 11*
Leaf	Air	256 ± 8	265 ± 13	237 ± 9
	N_2	248 ± 9	268 ± 18	296 ± 14*

Table III. *ADH Activity in Detached Leaves and Roots of Cottonwood*

Roots of intact plants were bubbled with air for 24 h before the start of the experiment. At time = 0, leaves and roots were detached and incubated in separatory funnels purged with air or with N_2. Details as in Table I.

		ADH Activity at Time		
		0	24	48 h
		μmol NADH oxidized/min^{-1}·g^{-1} dry wt		
Root	Air	48 ± 8	35 ± 4	28 ± 2
	N_2	38 ± 4	286 ± 6*	246 ± 9*
Leaf	Air	232 ± 8	246 ± 11	228 ± 14
	N_2	234 ± 6	258 ± 12	265 ± 22*

continually induced by substrate, such as: (a) acetaldehyde or ethanol delivered in the transpiration stream, or (b) other aldehydes or alcohols, such as hexenal, produced by lipid peroxidation (18).

The evidence is against these two alternatives. In the first instance, we would expect the leaf ADH activity to decrease in plants whose roots were bubbled with air, or in detached, air-purged leaves. In both of these experiments, there should have been a large decline (probably to zero) in fermentation products delivered to the leaf. Bubbling of roots with air would reduce fermentation in roots and thus decrease delivery of acetaldehyde or ethanol to the transpiration stream. Detached leaves incubated aerobically contain no acetaldehyde or ethanol (10). There was, however, no decline in ADH activity under these conditions. While root ADH activity declined by 60% in 48 h upon exposure of roots of intact plants to air, leaf ADH activity did not change significantly (Table I). ADH activity of detached, air-incubated leaves also did not change significantly, while that of roots declined (Table III). Moreover, in a number of experiments, we have been unable to detect any acetaldehyde or ethanol in leaves of aerobic, unstressed cottonwood plants (RC MacDonald, and TW Kimmerer, unpublished data), suggesting that delivery of acetaldehyde or ethanol to leaves is minimal.

To test the second alternative, that other aldehydes could be responsible for maintenance of leaf ADH activity, the activity of the crude enzyme preparation was tested with a number of aldehydes. As a percent of activity with acetaldehyde as substrate, ADH activity with other substrates was: formaldehyde, 5%; propionaldehyde, 1.5%; *trans*-2-hexenal, no detectable activity. A fuller test of a variety of substrates, and determination of apparent K_ms will await purification of the leaf enzyme. A further argument against this alternative is the presence of PDC activity in leaves, which is unlikely to play any role in metabolism of other aldehydes.

At present, we do not know the role(s) of leaf PDC and ADH. The enzymes are apparently constitutive in the leaves of cottonwood, and appear to occur at high activity in the leaves of many trees and shrubs, and at low activity in herbaceous plant leaves (10). Yet, acetaldehyde and ethanol are not usually produced by leaves except when they are stressed, particularly by air pollutants (9). ADH and PDC in leaves would appear to be without function most of the time, playing a role only in stressed plants.

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