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# Acetaldehyde and Ethanol Biosynthesis in Leaves of Plants[1]

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## ABSTRACT

**Leaves of terrestrial plants are aerobic organs, and are not usually considered to possess the enzymes necessary for biosynthesis of ethanol, a product of anaerobic fermentation. We examined the ability of leaves of a number of plant species to produce acetaldehyde and ethanol anaerobically, by incubating detached leaves in $N_2$ and measuring headspace acetaldehyde and ethanol vapors. Greenhouse-grown maize and soybean leaves produced little or no acetaldehyde or ethanol, while leaves of several species of greenhouse-grown woody plants produced up to 241 nanograms per milliliter headspace ethanol in 24 hours, corresponding to a liquid-phase concentration of up to 3 milligrams per gram dry weight. When leaves of 50 plant species were collected in the field and incubated in $N_2$, all higher plants produced acetaldehyde and ethanol, with woody plants generally producing greater amounts (up to 1 microgram per milliliter headspace ethanol concentration). Maize and soybean leaves from the field produced both acetaldehyde and ethanol. Production of fermentation products was not due to phylloplane microbial activity: surface sterilized leaves produced as much acetaldehyde and ethanol as did unsterilized controls. There was no relationship between site flooding and foliar ethanol biosynthesis: silver maple and cottonwood from upland sites produced as much acetaldehyde and ethanol anaerobically as did plants from flooded bottomland sites. There was no relationship between flood tolerance of a species and ethanol biosynthesis rates: for example, the flood intolerant species *Quercus rubra* and the flood tolerant species *Quercus palustris* produced similar amounts of ethanol. Cottonwood leaves produced more ethanol than did roots, in both headspace and enzymatic assays. These results suggest a paradox: that the plant organ least likely to be exposed to anoxia or hypoxia is rich in the enzymes necessary for fermentation.**

Leaves of terrestrial higher plants are quintessentially aerobic organs, adapted for high rates of gas exchange in an atmosphere rich in $O_2$. Dark respiration in leaves occurs via the tricarboxylic acid cycle (6, 15, 19). There is no apparent need in leaves for enzymes of anaerobic metabolism, and there are numerous reports that such enzymes and their mRNAs are absent and are not inducible in leaves (9, 13, 14, 18).

In roots, glycolysis leads to ethanol biosynthesis under anaerobic conditions via pyruvate, with concomitant oxidation of NADH, as a result of the action of PDC[2] and ADH (5). This is the only route of ethanol synthesis known in higher plants. This pathway, and the associate enzymes, have not been shown to occur in leaves, and the ADH1 gene has been shown to be totally repressed in maize leaves (14).

We have found, however, that leaves of some woody plants are capable of aerobic ethanol production upon exposure to $SO_2$, an atmospheric pollutant, and to certain other stresses (12), suggesting that woody plant leaves may contain PDC and ADH. We have now surveyed a large number of plant species for their ability to produce foliar acetaldehyde and ethanol under anaerobic conditions, as an *in vivo* test of the presence of PDC and ADH. We compared rates of acetaldehyde and ethanol synthesis by leaves of flood-tolerant and -intolerant plants, and by leaves from trees on flooded and upland sites. Flooding could induce ADH (though probably not PDC) if acetaldehyde or ethanol were translocated to the leaves in the transpiration stream. We also compared acetaldehyde and ethanol synthesis in anaerobically treated leaves and roots of *Populus deltoides*. In a subsequent paper (10) ADH activity in leaves and roots of cottonwood and soybeans are compared.

## MATERIALS AND METHODS

**Plant Materials—Greenhouse.** All plants except eastern cottonwood (*Populus deltoides* Bartr.) were grown from seed in 3:2:1 peat:perlite:vermiculite in Tree-Pots (Zarn Inc., Reidsville, NC) or Spencer-LeMaire bookplanters (Spencer-LeMaire Industries, Edmonton, Alberta). The soil mix was supplemented with controlled release fertilizers and plants were watered daily. The photoperiod was extended to 20 h with incandescent lamps, but no supplemental photosynthetic lighting was provided. The extended photoperiod maintained all plants in a state of free growth, with no bud set. Unless otherwise stated, the two youngest fully expanded leaves of each plant were used for assays. Leaf samples for headspace analysis were taken between 1000 and 1500 EDT.

Cottonwood cuttings (Clone K417) were rooted under mist and grown as above. The leaf plastochron index system of Larson and Isebrands (10) was applied to these cuttings. Unless otherwise stated, leaves with LPI 5 and 6 (the youngest fully expanded leaves) were used for assays.

The crop plant varieties used were: soybeans, *Glycine max* 'Pixie'; maize, *Zea mays* SX17A. Woody plants were grown from locally collected seed.

**Plant Material—Field.** Leaves of plants in farm fields, along hedgerows and in old-fields were collected in late August 1986

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[2] Abbreviations. PDC, pyruvic decarboxylase (EC 4.1.1.1); ADH, alcohol dehydrogenase (EC 1.1.1.1); LDH, lactic dehydrogenase (EC 1.1.1.27); $EtO_g$, $EtO_l$, vapor-phase and liquid-phase acetaldehyde, respectively; $EtOH_g$, $EtOH_l$, vapor-phase and liquid-phase ethanol, respectively.

in Garrard County, Kentucky and on University of Kentucky experimental farms in Fayette County, Kentucky. The youngest fully expanded leaves were harvested for assays. Senescent (yellowing) leaves were also collected from cottonwood and soybeans in the field to determine whether ethanol biosynthesis was senescence-related. Leaves were collected between 1100 and 1500 EDT, placed in plastic bags in a cooler at about 20°C, and transported to the laboratory. They were prepared for anaerobic treatment within 1 h after harvest.

**Headspace Assay for Acetaldehyde and Ethanol.** Leaves or roots were placed in 60-ml plastic syringes or glass jars and were then purged with air or $N_2$. Because headspace methods depend on concentration-dependent partition ratios between the gas phase and the liquid phase, tissue mass is not an important variable. Sufficient leaves were placed in incubation vessels to displace about 10 ml. In the field experiment, 5 g fresh weight of leaves were used for each replicate. Syringes or jars were sealed after purging, and periodic samples were injected into evacuated Teflon-stoppered tubes. The tube contents were analyzed by chromatography on a Varian 3700 GC with a flame ionization detector, using a DB-Wax 15 m × 0.53 mm column with a 1 μm film (J&W Scientific, Folsom, CA). GC conditions were: Column T, 50°C; Injector T, 110°C; Detector T, 150°C; He carrier gas flow, 5 ml/min; makeup $N_2$ flow, 25 ml/min. The electrometer signal was processed with a Varian DS-604 data system calibrated by injection of standard acetaldehyde and ethanol solutions in water, or by injection of a headspace sample from a Smith and Wesson (Eatontown, NJ) MK-II breath simulator. Identities of peaks were confirmed by GC-MS.

**Partition Ratios of Acetaldehyde and Ethanol.** Partition ratios for acetaldehyde and ethanol between air and water were determined empirically by sampling headspace vapors over well-stirred sealed containers with known concentrations of acetaldehyde and ethanol in water at 25°C.

**Enzymatic Assay for Ethanol.** The effectiveness of headspace ethanol assays as an estimator of plant ethanol liquid-phase concentration was tested by measuring headspace ethanol concentration around leaves and roots of $N_2$-purged cottonwood, then extracting ethanol from the tissues by homogenization in 100 mM glycine buffer (pH 9.0) at 4°C, centrifugation at 10,000*g* for 20 min at 4°C, and enzymatic determination of ethanol in the supernatant using the Sigma spectrophotometric procedure (Sigma Chemical Co.). Ethanol recovery did not change with short time periods after extraction provided that samples were maintained at 4°C, obviating the need for protein precipitation.

**Phylloplane Microbe Removal.** To determine whether phylloplane microbes might be partially responsible for acetaldehyde and ethanol synthesis in anaerobic leaves, the following procedure was used: field-collected soybean and cottonwood leaves were wiped with sterile filter paper moistened with sterile distilled water. The filter paper was incubated anaerobically and assayed for acetaldehyde and ethanol by the headspace method. Half of the leaves were surface sterilized by dipping in three changes of 0.1% Triton X-100 in sterile distilled water, followed by a 5 min soak in 1% Na hypochlorite. After rinsing, leaves were incubated for 4 h in syringes purged with air or $N_2$ and were then assayed for acetaldehyde and ethanol by the headspace method.

## RESULTS

**Headspace *versus* Liquid-Phase Acetaldehyde and Ethanol.** The partition ratios of acetaldehyde and ethanol between liquid and vapor phase exhibited quadratic isotherms (23°C) of the form:

$$ETO_g = 2.99\ (ETO_l) - 0.12\ (ETO_l)^2$$

$$ETOH_g = 0.20\ (ETOH_l) - 9.15 \times 10^{-3}\ (ETOH_l)^2$$

where vapor-phase concentrations are in μg/ml, and liquid-phase concentrations are in mg/ml. A headspace concentration of 1 μg ETOH/ml would thus correspond to a liquid-phase concentration of 7.75 mg/ml. Similarly, a headspace acetaldehyde concentration of 1 μg/ml would correspond to a liquid-phase concentration of 340 μg/ml. Because partition ratios depend on concentration, and not on mass or volume, the amount of leaf tissue is not a critical variable.

**Species Survey—Greenhouse Plants.** Greenhouse-grown maize and soybean leaves were not competent to produce acetaldehyde or ethanol, while all woody plants examined produced large amounts of both compounds (Table I). When we compared rates of synthesis of ethanol over time following the onset of anoxia, three kinds of responses were observed (Fig. 1): (a) immediate, rapid ethanol synthesis (*Populus, Quercus*); (b) ethanol synthesis following a 2 to 6 h lag (*Pinus, Fraxinus, Acer*); and (c) no ethanol synthesis (*Zea, Glycine*). Aerobic control leaves produced no detectable acetaldehyde or ethanol. Leaf samples taken early in the photoperiod were often low in anaerobic acetaldehyde and ethanol synthesis. Sampling for these experiments was restricted to 1000 to 1500 EDT.

**Species Survey—Field Plants.** Leaves of the majority of plants sampled were capable of producing at least some acetaldehyde and ethanol under anaerobic conditions, with woody plants generally producing large amounts (Table II). Ferns produced no

Table I. *Acetaldehyde and Ethanol Production by Leaves of Greenhouse-grown Plants after 24 h Incubation in Anaerobic Conditions*

Data are mean ± SE headspace acetaldehyde or ethanol concentration. Two or three leaves were sampled from each of three plants. Aerobic controls produced trace amounts of ethanol and no acetaldehyde.

| Species | Acetaldehyde | Ethanol |
|---|---|---|
| | *ng/ml* | |
| Woody plants | | |
| *Quercus alba* | 35 ± 9 | 175 ± 76 |
| *Liquidambar styraciflua* | 53 ± 26 | 241 ± 97 |
| *Fraxinus americana* | 26 ± 8 | 227 ± 110 |
| *Fraxinus pennsylvanica* | 32 ± 25 | 188 ± 117 |
| *Populus deltoides* | 130 ± 63 | 187 ± 43 |
| *Pinus taeda* | 10 ± 10 | 226 ± 13 |
| Herbaceous plants | | |
| *Zea mays* | Tr[a] | Tr |
| *Glycine max* | 0 | Tr |

[a] Tr = Trace (<1 ng/ml), 0 = not detectable (<0.05 ng/ml).

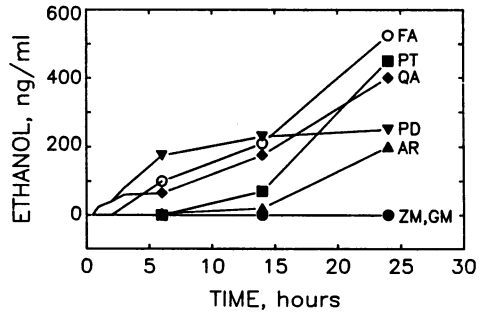


FIG. 1. Headspace ethanol from leaves of 7 species of plants grown in the greenhouse. Leaves were placed in jars and purged with $N_2$ at Time = 0, with 1 to 3 leaves per jar, and 3 replicates of one plant each. Data are means; SE were ≤15% of the mean. Data symbols are omitted for the first 3 h for clarity. Control leaves incubated in air-purged jars produced no detectable acetaldehyde or ethanol. Woody species were FA—*F. americana*, PT—*P. taeda*, QA—*Q. alba*, PD—*P. deltoides*, AR—*A. rubrum*. Herbaceous plant species were ZM—*Z. mays*, GM—*G. max*.

Table II. *Acetaldehyde and Ethanol in Headspace over Plant Leaves Incubated in $N_2$*

Air-purged control leaves produced no detectable acetaldehyde or ethanol. Data are mean ± SE of 3 replicate 5-g leaf samples.

| Species | 4-h Incubations | | 24-h Incubations | |
|---|---|---|---|---|
| | Acetaldehyde | Ethanol | Acetaldehyde | Ethanol |
| | *ng/ml* | | | |
| A. Herbaceous plants | | | | |
| *Lycopodium digitatum* A. Braun | 0 ± 0 | 0 ± 0 | 0 ± 0 | 0 ± 0 |
| *Equisetum arvense* L. | 0 ± 0 | 0 ± 0 | 0 ± 0 | 0 ± 0 |
| Ferns (5 species)[a] | 0 ± 0 | 0 ± 0 | 0 ± 0 | 0 ± 0 |
| *Vicia sativa* L. | 11 ± 3 | 6 ± 1 | 45 ± 13 | 2 ± 0 |
| *Zinnia* sp. | 9 ± 5 | 9 ± 3 | 0 ± 0 | 10 ± 3 |
| *Arctium minus* (Hill) Bernh. | 5 ± 2 | 14 ± 4 | 17 ± 3 | 16 ± 1 |
| *Ambrosia artemisiifolia* L. | 4 ± 0 | 56 ± 1 | 7 ± 0 | 17 ± 17 |
| *Phytolacca americana* L. | 5 ± 0 | 23 ± 1 | 0 ± 0 | 22 ± 8 |
| *Glecoma hederacea* L. | 0 ± 0 | 5 ± 5 | 16 ± 0 | 22 ± 2 |
| *Trifolium pratense* L. | 0 ± 0 | 31 ± 4 | 0 ± 0 | 36 ± 3 |
| *Fragaria virginiana* Duchesne | 48 ± 9 | 38 ± 13 | 38 ± 8 | 46 ± 7 |
| *Plantago major* L. | 0 ± 0 | 30 ± 4 | 6 ± 1 | 57 ± 10 |
| *Glycine max* L. | 0 ± 0 | 26 ± 3 | 5 ± 0 | 62 ± 7 |
| *Setaria viridis* (L.) Beauvois | 0 ± 0 | 2 ± 0 | 0 ± 0 | 67 ± 12 |
| *Zea mays* L. | 0 ± 0 | 40 ± 2 | 8 ± 3 | 155 ± 6 |
| *Solidago* sp. | 18 ± 1 | 170 ± 15 | 0 ± 0 | 245 ± 74 |
| *Asclepias verticillata* L. | 0 ± 0 | 63 ± 2 | 9 ± 4 | 277 ± 20 |
| Mean ± SE for all herbaceous plants | 5 ± 2 | 24 ± 8 | 7 ± 3 | 49 ± 17 |
| B. Woody plants | | | | |
| *Robinia pseudoacacia* L. | 0 ± 0 | 127 ± 13 | 0 ± 0 | 103 ± 19 |
| *Thuja occidentalis* L. | 0 ± 0 | 46 ± 4 | 0 ± 0 | 114 ± 3 |
| *Ginkgo biloba* L. | 41 ± 0 | 56 ± 20 | 117 ± 53 | 123 ± 4 |
| *Magnolia acuminata* L. | 5 ± 1 | 68 ± 4 | 2 ± 2 | 195 ± 74 |
| *Cercis canadensis* L. | 6 ± 6 | 119 ± 8 | 0 ± 0 | 202 ± 94 |
| *Aesculus octandra* Marsh. | 0 ± 0 | 52 ± 7 | 0 ± 0 | 226 ± 7 |
| *Juniperus virginiana* L. | 6 ± 1 | 64 ± 2 | 18 ± 6 | 233 ± 3 |
| *Campsis radicans* L. | 4 ± 0 | 56 ± 1 | 7 ± 0 | 251 ± 4 |
| *Vitis* sp. | 7 ± 1 | 93 ± 12 | 17 ± 4 | 277 ± 48 |
| *Picea pungens* Engelm. | 0 ± 0 | 11 ± 3 | 0 ± 0 | 298 ± 19 |
| *Malus pumila* L. | 9 ± 1 | 132 ± 6 | 0 ± 0 | 335 ± 34 |
| *Juglans nigra* L. | 0 ± 0 | 120 ± 67 | 0 ± 0 | 342 ± 3 |
| *Platanus occidentalis* L. | 0 ± 0 | 79 ± 8 | 0 ± 0 | 354 ± 24 |
| *Gleditsia triacanthos* L. | 3 ± 3 | 149 ± 45 | 14 ± 15 | 382 ± 1 |
| *Celtis occidentalis* L. | 2 ± 2 | 94 ± 29 | 0 ± 0 | 407 ± 69 |
| *Lonicera* sp. | 21 ± 2 | 77 ± 2 | 40 ± 4 | 413 ± 66 |
| *Prunus serotina* Ehrh. | 4 ± 0 | 99 ± 13 | 6 ± 1 | 438 ± 30 |
| *Tsuga canadensis* (L.) Carr | 16 ± 2 | 62 ± 4 | 26 ± 6 | 491 ± 49 |
| *Larix decidua* Mill | 0 ± 0 | 71 ± 4 | 0 ± 0 | 536 ± 15 |
| *Liquidambar styraciflua* L. | 5 ± 5 | 145 ± 16 | 0 ± 0 | 593 ± 163 |
| *Quercus alba* L. | 5 ± 0 | 97 ± 1 | 12 ± 12 | 606 ± 67 |
| *Liriodendron tulipifera* L. | 12 ± 1 | 87 ± 1 | 14 ± 14 | 723 ± 68 |
| *Pinus strobus* L. | 0 ± 0 | 89 ± 19 | 0 ± 0 | 734 ± 3 |
| *Rubus* sp. | 16 ± 1 | 141 ± 20 | 24 ± 0 | 946 ± 144 |
| *Quercus rubra* L. | 0 ± 0 | 157 ± 12 | 0 ± 0 | 960 ± 76 |
| *Quercus palustris* Muenchh. | 0 ± 0 | 172 ± 51 | 0 ± 0 | 999 ± 17 |
| *Betula lenta* L. | 0 ± 0 | 193 ± 6 | 29 ± 3 | 1051 ± 47 |
| Mean ± SE for all woody plants | 6 ± 2 | 91 ± 8 | 11 ± 4 | 435 ± 50 |

[a] The 5 fern species tested were: *Pteridium aquilinum* L., *Onoclea sensibilis* L., *Osmunda cinnamonea* L., *Polystichum acrostichoides* (Michx.) Schott, *Asplenium rhizophyllum* L. Mean ± SE is for 5 species, 3 replicates per species.

acetaldehyde or ethanol, and most herbaceous plants produced relatively small amounts of acetaldehyde and ethanol. Leaves of both maize and soybean collected in the field were competent to produce acetaldehyde and ethanol, with maize leaves producing 155 ng/ml headspace ethanol by 24 h. The other herbaceous plants with high ethanol production were goldenrod (*Solidago* sp.) and milkweed (*Asclepias major*). All other herbaceous plants produced less than 70 ng/ml headspace ethanol at 24 h, while all woody plants tested produced in excess of 100 ng/ml headspace ethanol at 24 h. Several conifers produced very low amounts of ethanol at 4 h but large amounts at 24 h, suggesting an induced production of ethanol, as shown for pine in the greenhouse study (Fig. 1). For example, *Picea pungens* (Table II) had produced only 11 ng/ml headspace ethanol by 4 h, but had

produced 298 ng/ml headspace ethanol by 24 h. Aerobic control leaves of all species produced no detectable acetaldehyde or ethanol.

There was little difference in acetaldehyde production between woody and herbaceous plants in the field study (Table II), and little correlation between acetaldehyde and ethanol production. A few plants (*e.g. Fragaria virginiana* and *Ginkgo biloba*) produced fairly large amounts of acetaldehyde, and headspace vapors around the leaves had a distinct acetaldehyde odor. Many plants which produced large amounts of ethanol had little or no detectable acetaldehyde (*e.g. Quercus palustris* and *Pinus strobus*).

After the 24 h incubation period, leaves were inspected for necrosis. Fern leaves, which produced no acetaldehyde or ethanol, were flaccid and had water-soaked lesions. They became brown after about 10 min exposure to air. Similarly, *Vicia coronata* leaves had necrotic areas which became brown. Leaves of other species did not appear to be necrotic, although many were flaccid.

There was no relationship between site flooding and ability of leaves to produce ethanol anaerobically. We compared cottonwood and silver maple leaves on flooded river-bottom sites and dry upland sites, and found no significant effect of flooding on foliar acetaldehyde or ethanol production (Table III). Aerobic controls produced no detectable acetaldehyde or ethanol. Senescent leaves of soybean and cottonwood produced little acetaldehyde or ethanol anaerobically, and there was a direct correlation between remaining green tissue and ethanol production (data not shown).

**Phylloplane Microbes.** We wondered whether phylloplane microbial activity could have produced some of the fermentation products observed, particularly in view of the differences between field and greenhouse maize and soybean. Surface-sterilized leaves of field-grown plants produced as much acetaldehyde and ethanol under anaerobic conditions as did unsterilized leaves.

Surface-sterilized and unsterilized control cottonwood leaves produced 318 ± 53 ng/ml and 331 ± 48 ng/ml headspace ethanol, respectively. Surface-sterilized and unsterilized control soybean leaves produced 82 ± 26 and 88 ± 16 ng/ml headspace ethanol, respectively. Differences between treatments in acetaldehyde production were not significant. Filter paper wiped across the leaves did not produce any detectable acetaldehyde or ethanol under anaerobic conditions. We have cultured cottonwood leaves, sterilized by the method described, on nutrient agar to produce tissue cultures, and have not observed microbial contamination.

**Acetaldehyde and Ethanol Synthesis by Cottonwood Leaves and Roots.** We compared the rates of production of acetaldehyde and ethanol in greenhouse-grown cottonwood leaves and roots by headspace assay (Fig. 2). Ethanol release from leaves was much greater than from roots, while aerobic controls produced no detectable ethanol (Fig. 2A). Ethanol production by leaves had quadratic kinetics, reaching a maximum by 13 h. This was not due to saturation of the air with vapor-phase ethanol, since the partition equations (above) predict saturation to occur at a vapor-phase concentration in excess of 1 μg/ml. Acetaldehyde was similarly at a low concentration in headspace around roots, and much higher in headspace around leaves (Fig. 2B).

Headspace assays could underestimate ethanol production if ethanol release from leaves and roots is diffusion limited. This is more likely for roots, with their low surface-to-volume ratios. We compared ethanol in anaerobic leaves and roots by headspace assay and by enzymic assay of leaf and root homogenates (Table IV). While the headspace assay underestimated the amounts of ethanol in leaves and roots, there was little difference in the efficiency of the headspace assay between leaves and roots.

Table III. *Acetaldehyde and Ethanol Production by Anaerobic Leaves of Two Flood Tolerant Tree Species, Silver Maple (Acer saccharinum) and Cottonwood (Populus deltoides)*

Samples were taken on the same day from a river bottom site and two nearby upland sites on well-drained soils in Grant County, Kentucky. Aerobic leaves produced no detectable acetaldehyde or ethanol. Data are mean ± SE headspace concentration for 10 trees from each species and site, 1 leaf per sample.

| | Acetaldehyde | | Ethanol | |
|---|---|---|---|---|
| | 4 h | 24 h | 4 h | 24 h |
| | *ng/ml* | | | |
| *Populus deltoides* | | | | |
| Flooded site | 16 ± 9 | 156 ± 18 | 135 ± 24 | 460 ± 36 |
| Upland site | 8 ± 2 | 164 ± 31 | 186 ± 18 | 582 ± 42 |
| *Acer saccharinum* | | | | |
| Flooded site | 48 ± 14 | 78 ± 12 | 78 ± 12 | 426 ± 54 |
| Upland site | 62 ± 12 | 102 ± 18 | 64 ± 18 | 411 ± 21 |

## DISCUSSION

The copious production of ethanol by leaves of many plants suggest a biochemical and genetic paradox: that the plant organ

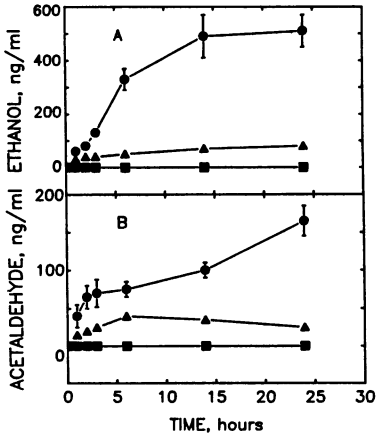


FIG. 2. Acetaldehyde (A) and ethanol (B) production by leaves and roots of cottonwood. Leaves were incubated in jars in air or $N_2$ and samples were taken periodically for headspace assay. Data are mean ± SE for 1 leaf or root from each of 6 plants. Air controls (■) are for both leaf and root, which produced only trace amounts of either acetaldehyde or ethanol. Anaerobic roots (▲) produced significantly ($P < 0.01$, rank test) more acetaldehyde and ethanol than did controls, as did anaerobic leaves (●).

Table IV. *Comparison of Enzymic and Headspace Assay for Ethanol in Cottonwood*

Ethanol concentration (μg EtOH/g dry weight) was converted to liquid-phase concentration (μg EtOH/ml $H_2O$) by

$$\frac{\mu g\ EtOH}{ml\ H_2O} = \frac{\mu g\ EtOH}{g\ dry\ weight} \times \frac{g\ dry\ weight}{g\ fresh\ weight - g\ dry\ weight}$$

Headspace ethanol concentration was converted to liquid-phase concentration using the conversion factor given in the text. Data are mean ± SE for measurements of independent samples of leaves or roots from three plants incubated in $N_2$ for 6 h.

| | Ethanol Concentration | | |
|---|---|---|---|
| | Enzymatic Assay | | GC Assay |
| | *mg/g dry weight* | *μg/ml* | *μg/ml* |
| Leaf | 3.83 ± 0.13 | 1017.9 ± 38.5 | 589.1 ± 38.2 |
| Root | 2.15 ± 0.43 | 102.6 ± 21.1 | 56.0 ± 3.6 |

least likely to be exposed to anoxia or hypoxia is rich in the enzymes necessary for fermentation. Our results are in marked contrast to previous studies, which demonstrated a lack of the necessary enzymes in leaves and an inability of leaves exposed to anoxia to produce anaerobic proteins or polypeptides, and their associated mRNAs (7, 9, 14). These previous experiments were all done with greenhouse or growth-chamber plants. We, too, found no evidence that maize or soybean leaves could synthesize ethanol when grown in the greenhouse or growth chamber. However, leaves of greenhouse-grown woody plants, and of a wide variety of plants collected in the field, including maize and soybean, were competent to produce ethanol when placed in an anaerobic environment. These data, particularly the comparison of greenhouse- and field-grown maize and soybeans, suggest that under some circumstances, leaves of these plants can be induced to synthesize the necessary enzymes.

We observed three patterns of response to anoxia in leaves of greenhouse-grown plants (Fig. 1): (a) immediate ethanol synthesis, (b) ethanol synthesis after a lag, and (c) no synthesis. These responses are suggestive of three patterns in the biochemical pathway: (a) constitutive enzyme system, (b) inducible enzyme system, and (c) enzyme system repressed. While we do not presently know the pathway of foliar ethanol synthesis for certain, we have found ADH and PDC activity in cottonwood leaves (11), and no other route to ethanol synthesis is known in higher plants.

We found maximal headspace ethanol concentrations of up to 1 $\mu$g/ml after 24 h incubation of leaves in $N_2$, corresponding to liquid-phase concentrations of up to 7.75 mg/ml using our empirical conversion, which assumes that the liquid phase of the plant is pure, free water. The headspace assay underestimated ethanol concentration when compared with the enzymic assay, which may be due to the effect of solutes on the partition ratio between liquid and vapor phase. Similar results were observed in comparing liquid/vapor partition ratios of ethanol dissolved in water, urine, or blood (8). The headspace assay has the advantages of simplicity and speed, and allows the simultaneous determination of ethanol, acetaldehyde, ethylene, and ethane (12).

Others (1) have suggested that the ethanol levels in our previous experiments (12) were very low, reflecting low activities of foliar ADH. However, in order to compare results, it is necessary to take into account the difference between our headspace estimates and liquid-phase concentrations. Most previous studies (*e.g.* Ref. 1), used enzymic or other liquid-phase assays. Our results for roots are quite consistent with reported data, either by enzymic analysis (Table IV), or by conversion of vapor-phase to liquid-phase concentrations using the above equations. Moreover, we found that the foliar ethanol concentration following anaerobic incubation was considerably higher than that of roots, regardless of the assay method employed (Table IV). In all of our experiments, cottonwood leaves produced more ethanol than did roots.

Acetaldehyde and ethanol production were not well correlated. While no plants produced acetaldehyde without also producing ethanol, many plants produced abundant ethanol without detectable acetaldehyde production (Table II). Acetaldehyde is considerably more toxic to plants than is ethanol (4, 15), and maintenance of higher activity of ADH than of PDC may be important to the survival of plant cells which become anaerobic. While a few plants produced almost as much headspace acetaldehyde as ethanol (Table II), this should not be taken as an indication of low ADH activity: the vapor pressure of acetaldehyde is considerably higher than is that of ethanol. For example, leaves of *Ginkgo biloba* produced 118 ng/ml acetaldehyde and 123 ng/ml, respectively. This corresponds to liquid-phase concentrations of acetaldehyde and ethanol of 90 and 650 ng/ml, respectively. *Vicia coronata* (Table II) was the only species for which acetaldehyde production exceeded ethanol production when the gas-liquid partition ratios are taken into account. Leaves of this plant were partially necrotic at the end of the 24 h incubation.

There was little correlation between native habitat and foliar acetaldehyde or ethanol production under anaerobic conditions. For example, the flood-tolerant *Quercus palustris* produced no more ethanol than did the flood-intolerant *Quercus rubra*, and the flood-tolerant *Betula nigra* produced significantly less ethanol than did the flood-intolerant *Betula lenta* (Table II). These trees were growing on upland sites with similar soil texture. Trees on upland and bottomland sites synthesized similar amounts of acetaldehyde and ethanol anaerobically (Table III), and produced no detectable acetaldehyde or ethanol aerobically. Foliar ethanol biosynthesis, and the presence of PDC and ADH in leaves (11), appear to be unrelated to flood tolerance or site flooding. If foliar ADH played a role in metabolizing xylem-translocated fermentation products, we would expect higher activity (*i.e.* greater anaerobic ethanol synthesis) in leaves from flooded plants or from flood-tolerant plants. This was not the case.

Ethanol production was not due to phylloplane microbial contamination. We believe our results can best be explained by the occurrence of both PDC and ADH in leaves of most plants as constitutive or inducible enzyme systems. We have found high ADH activity and have detected PDC activity in cottonwood leaves (11). These are not the only enzymes associated with anaerobiosis which have been found in leaves: Betsche (2, 3) found LDH in leaves of several plant species, including soybean, and suggested that foliar LDH may be part of a metabolic pH-stat. Roberts *et al.* (16, 17) have demonstrated that transient organic acid formation triggers ethanol biosynthesis in hypoxic root tips by causing a drop in cytoplasmic pH. This acidosis is then relieved by ethanol synthesis. Ethanol biosynthesis could thus be viewed as a mechanism to avoid acidosis, rather than to avoid anaerobiosis per se. It is of particular interest to note that ethanol synthesis occurs in leaves under aerobic conditions when they are stressed by sulfur dioxide, an acidic gas (12).

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