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Malic Acid Accumulation in Roots in Response to Flooding: Evidence Contrary to its Role as an Alternative to Ethanol

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ABSTRACT

Across a time course of flooding the malic acid content in roots of the swamp tree *Nyssa sylvatica* var. *biflora* increased 5-fold from 1 week to 1 month of flooding and remained at that level through a full year of flooding. Alcoholic fermentation rates accelerated within the first month of flooding but dropped to very low levels under long-term flooding. The theory that, under flooding, malic acid accumulates as an alternative anaerobic end product to ethanol is unlikely in this instance since (1) malate is initially associated with high alcoholic fermentation and (2) the reduction in alcoholic fermentation is accounted for by increased internal aeration of the roots.

INTRODUCTION

Dark fixation of CO_2 and accumulation of malic acid in roots has been implicated as a means of avoiding ethanol toxicity under the hypoxic conditions of water-logged soils (McManmon and Crawford, 1971). This is based on the observation that, after a short period of flooding, the roots of flood-tolerant species have low alcoholic fermentation rates coupled with a high accumulation of malic acid. Specifically, the roots of 'flood-tolerant' herbs do not show accelerated rates of ethanol production after a month of flooding (Crawford, 1966), but do show an increase in the concentration of malic acid after 4 d of flooding, and the pattern is quite the opposite for 'non-flood-tolerant' species (Crawford and Tyler, 1969); however, the ratio of malic acid/ethanol is variable after a month of flooding (Crawford, 1967). It has been hypothesized that malic acid is an alternative end product to ethanol in anaerobic respiration (Fig. 1); one which is non-toxic and can be temporarily stored and then metabolized on return to aerobic conditions.

The potential generality of this theory for explaining flood tolerance in many plant life-forms is suggested by more recent experiments on woody species. Crawford (1972) has shown that birch trees growing in wet soils have approximately twice the concentration of malic acid in their sap as ones growing in dry soils, although the sap ratio of malic acid/ethanol was approximately the same in both soils. Additionally, the roots of the flood-tolerant tree, *Pinus contorta* Dougl. ex Loud., showed only a 3-fold increase in ethanol content after 24 h of flooding, whereas the non-flood-tolerant *Picea sitchensis* (Bong.) Carr. showed a

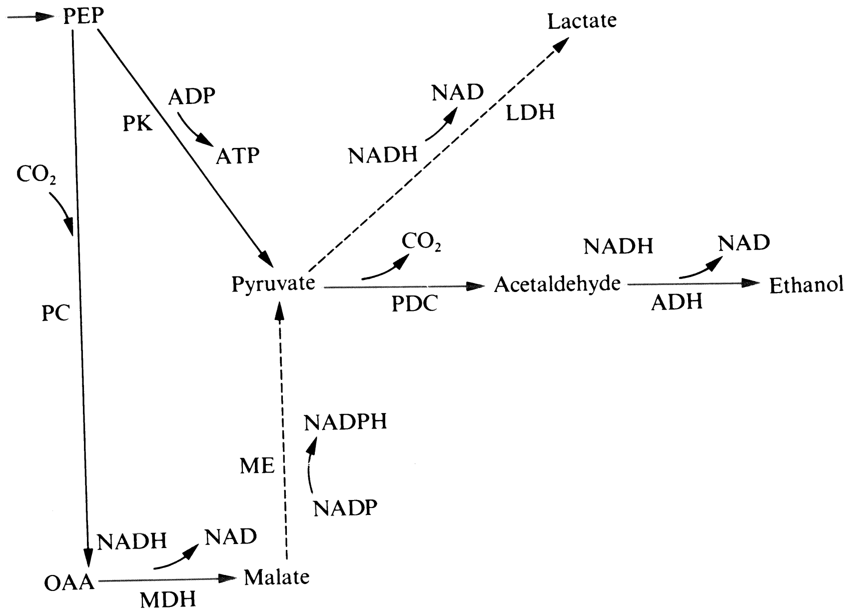

FIG. 1. Terminal stages of glycolysis and potential carbon pathways under low oxygen conditions. PEP, phosphoenolpyruvate; PK, pyruvate kinase; LDH, lactate dehydrogenase; PDC, pyruvate decarboxylase; ADH, alcohol dehydrogenase; PC, PEP carboxylase; MDH, malic dehydrogenase; ME, malic enzyme. (Dashed lines indicate pathways which are of doubtful importance in many plants.)

10-fold increase (Crawford and Baines, 1977), and after 4 months of flooding *Pinus contorta* had anaerobic respiration rates comparable to drained controls but *Picea sitchensis* had higher rates than drained controls (Crawford, 1976). Also, in this latter study it was shown that *Pinus contorta* roots fix CO_2 into malic acid at about the same rate under nitrogen or air whereas *Picea sitchensis* fixes less CO_2 into malic acid under nitrogen than under air.

Data are presented here for the swamp tree *Nyssa sylvatica* var. *biflora* (Walter) Sarg. which show a response similar in part to previous reports, i.e. high malic acid accumulation coupled with low rates of alcoholic fermentation after a period of flooding. However, the pattern of malic acid accumulation across a time course of flooding suggests that the previous interpretation does not hold for this species.

MATERIALS AND METHODS

Plants and treatments

One year old seedlings, grown from seed in a glasshouse, were used for all experiments. Seeds were collected from trees growing in swamps on the coastal plain of Georgia and South Carolina in the south-eastern United States. Following a cold treatment, seeds were germinated in 15 cm clay pots filled with sandy loam soil and the seedlings were supplied with half-strength Hoagland's solution every other week. Flooding treatment was imposed by placing the pots in a flood-tank filled with water to a level 3–5 cm above the top of the pots. Drained pots were watered lightly every other day during the winter and every day in the summer.

Analysis

Two measures of alcoholic fermentation were utilized. One was an *in vivo* assay of ethanol production in root segments under anaerobic incubation similar to that described by Crawford

(1966). The other was an *in vitro* assay of alcohol dehydrogenase (ADH; EC 1.1.1.1) activity in roots taken directly out of the soil.

Analytical procedures were as described previously (Keeley, 1977). Briefly, ethanol production was measured in 250 ml flasks with *c.* 1 g of 1 cm root sections in 30 ml 0·05 M citrate-0·01 M phosphate buffer (pH 5·4) with 0·25% glucose. After 4 h incubation, root sections were blotted and weighed fresh, then ground in a mortar and centrifuged. The supernatant was added to the incubation buffer and enzymatically assayed for ethanol (Bergmeyer, 1974). Since the moisture content of the roots varied significantly across treatments (Keeley, 1977) fresh/dry weight ratios were calculated for a large number of samples and used to convert the ethanol production to a dry weight basis.

Alcohol dehydrogenase activity was determined as follows. Root sections were ground in an ice bath and centrifuged at 2 °C and the clear supernatant was assayed for specific activity with acetaldehyde as substrate at 25 °C as described by Crawford (1967). The concentrations in the final cuvette were: 95 mM Tris buffer (pH 8·0), 66 mM NADH, *c.* 100 mg ml^{-1} protein, and 33 mM acetaldehyde. Protein was determined by the Lowry method (as modified by Bergmeyer, 1974) on acid precipitates.

Malic acid concentrations were determined as described by Crawford and Tyler (1969). Root sections were ground in an ice bath, centrifuged, and the clear supernatant assayed for malate by an enzymatic procedure (Bergmeyer, 1974).

Data were analysed with the one-way ANOVA using the fixed effects model testing main effects against error.

RESULTS AND DISCUSSION

The flood-tolerant *Nyssa sylvatica* accelerates its ethanol producing capacity under short-term flooding but decelerates it under long-term flooding (Table 1). This parallels changes in alcohol dehydrogenase activity and illustrates a more dynamic relationship between alcoholic fermentation rates and malic acid accumulation (Fig. 2) than previously described. Two points argue against the role of malic acid as an alternative anaerobic end product to ethanol in *Nyssa sylvatica* var. *biflora.*

TABLE 1. *Ethanol production rates (μmol h^{-1} g^{-1} dry wt.) under anaerobic incubation in excised roots from 1 year old seedlings*

Drained	Flooded			
	1 week	1 month	1 year	
22·7 ± 2·9[a] ($n = 4$)	42·3 ± 2·7 ($n = 2$)	57·7 ± 12·0 ($n = 2$)	13·0 ± 1·5 ($n = 4$)	$F_s = 14{\cdot}50$***

[a] ± one s.e. mean.
*** $P < 0{\cdot}001$.

1. Malic acid accumulation is not always associated with low alcoholic fermentation rates. After 1 month of flooding ethanol production rates, ADH activity, and malic acid content are all high; evidence that is difficult to reconcile with the theory that malate is accumulating as a result of a metabolic switch away from ethanol production.

2. The low alcoholic fermentation rate after a year of flooding is due to a shift to aerobic respiration, arising from a greatly increased internal oxygen supply to the roots (Keeley and Franz, 1978). Thus it appears that malate is not only accumu-

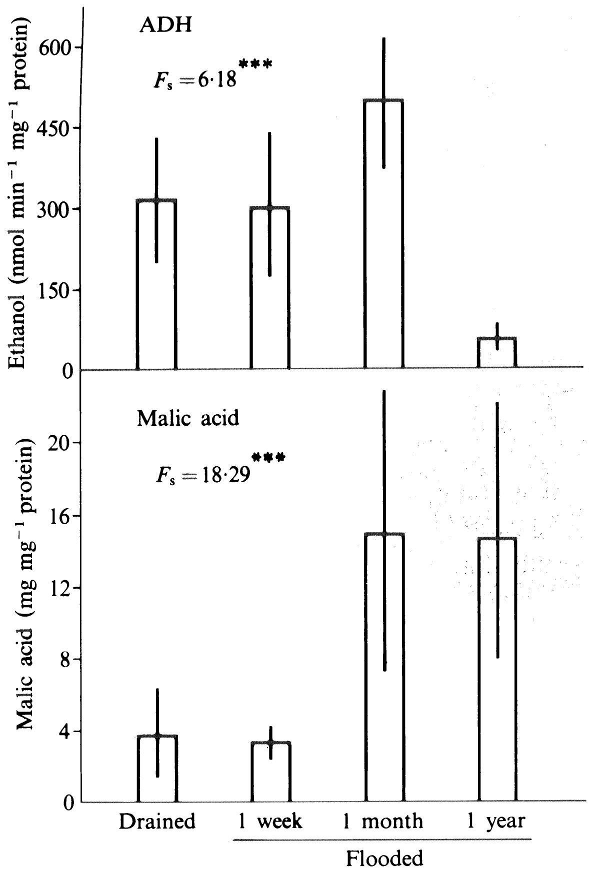

FIG. 2. Specific activity of alcohol dehydrogenase (ADH) for acetaldehyde and malic acid concentration in the roots of seedlings of the same age grown under drained conditions and across a time course of flooding; means, 95% confidence limits, and F statistics for one-way ANOVA ($*** = P < 0{\cdot}001$). Sample sizes for ADH and malic acid respectively are: drained (9, 5), flooded 1 week (12, 10), 1 month (15, 15), and 1 year (16, 15).

lating independently of alcoholic fermentation but that it is also accumulating under relatively aerated conditions within the root.

An hypothesis currently being investigated is that malate accumulates in response to ionic disequilibrium in the roots. This is suggested by numerous studies showing roots increase their malic acid content when cations are absorbed in excess of anions, and this can be induced by an increased cation concentration in the rooting medium (Jacobson and Ordin, 1954; Jacobson, 1965; Hiatt, 1967; Jacoby and Laties, 1971). The idea is worth considering, since, upon flooding, several soil characteristics are radically affected, e.g. certain cations such as the divalent form of manganese can increase by orders of magnitude (Bould and Hewitt, 1963). Since it is this base-exchangeable form of manganese which is taken up by plants (Quastel, 1963), species in flooded soils typically accumulate large concentrations of manganese, as did the plants used in the present study (Keeley, 1977). This would be just one of numerous ways the ionic balance of a cell could be upset under flooded conditions.

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