Ethanol-Induced Injuries to Carrot Cells$^{1,2}$

The Role of Acetaldehyde

Pierdomenico Perata and Amedeo Alpi*

*Dipartimento di Biologia delle Piante Agrarie, Università degli Studi di Pisa, Viale delle Piagge 23, 56124 Pisa, Italy

ABSTRACT

Carrot (*Daucus carota* L.) cell cultures show high sensitivity to ethanol since both unorganized cell growth and somatic embryogenesis are strongly inhibited by ethanol at relatively low concentrations (10–20 millimolar). The role of acetaldehyde on ethanol-induced injuries to suspension cultured carrot cells was evaluated. When ethanol oxidation to acetaldehyde is prevented by adding an alcohol-dehydrogenase (EC 1.1.1.1) inhibitor (4-methylpyrazole) to the culture medium, no ethanol toxicity was observed, even if ethanol was present at relatively high concentrations (40–80 millimolar). Data are also presented on the effects of exogenously added acetaldehyde on both carrot cell growth and somatic embryogenesis. We conclude that the observed toxic effects of ethanol cannot be ascribed to ethanol per se but to acetaldehyde.

On the basis of experimental results and after a reexamination of the literature, Jackson *et al.* (10) concluded that, “despite a widespread belief to the contrary, ethanol plays only a minor role in flooding injury to roots and shoots.” It had been generally believed that ethanol produced by plant tissues deprived of oxygen was highly toxic (for a review see ref. 8) and therefore could play an important role in causing anoxia injuries. However, Jackson *et al.* (10) applied ethanol to pea plants, detached leaves, and leaf protoplasts and none of these experiments showed toxic effects even when the ethanol concentrations were higher than those commonly found in anoxia-stressed pea plants. We (2) subsequently confirmed Jackson and coworkers’ (10) findings that ethanol exogenously applied to cereal seedlings was unable to mimic the toxic effects of anoxia.

Nevertheless, 1 year later (15) we found plant systems showing high sensitivity to the presence of ethanol even at low concentrations comparable to those commonly found in anoxia-stressed plant tissues. For instance, the growth of *Helianthus tuberosus* discs grown *in vitro* was inhibited more than 50% by 8.5 mM ethanol, induction of α-amylase by GAs in barley aleurone layers was strongly reduced by 34 mM ethanol, and the process of carrot somatic embryogenesis showed high sensitivity to ethanol being almost arrested if 10 mM ethanol was present in the culture medium.

The effects of ethanol on carrot cell cultures were further investigated (16). The results indicated that not only carrot somatic embryogenesis but also carrot cell growth was influenced by the presence of ethanol, though at higher concentrations, and that carrot cell cultures produce and release ethanol in the culture medium at concentrations comparable to those inducing toxicity.

Recently, a possible role for acetaldehyde in inducing hepatic injury following ethanol ingestion (7, 20) has been suggested, and the occurrence of conjugation products between acetaldehyde and erythrocyte or plasma proteins has been reported (9).

At present, no data have been published on the effects of acetaldehyde on plants even though acetaldehyde production has been reported in several plant species (11). Our aim is to investigate whether the observed toxic effects of ethanol on carrot cells can be ascribed to ethanol per se or to its oxidation to acetaldehyde.

MATERIALS AND METHODS

Plant Material and Culture Conditions

Carrot cells (*Daucus carota* L.) were grown in suspension in Gamborg’s BS medium, purchased as powder (Flow laboratories, Irvine, Ayshire, UK). The growth medium was supplemented with 0.5 mg/L 2,4-D (BDH, Poole, Dorset, UK) and 0.25 mg/L 6-benzylaminopurine (Sigma, St. Louis, MO).

Subculturing was performed every 20 d by inoculating 1 mL packed cell volume (after centrifugation for 5 min at 200g) into 25 mL fresh medium.

Somatic embryogenesis was obtained, starting from the 50 to 120 μM fraction of the culture as described by Vergara *et al.* (19). The embryogenic medium was hormone free.

Culture conditions strongly influence the time at which endogenous ethanol production took place. In order to minimize endogenous ethanol production (so that only the exact amount of exogenously added ethanol was present in the culture medium) cell culture conditions were slightly modified from those previously described (16). Cultures were kept in flasks at 25°C on rotary shakers (120 rpm) under a light intensity of 200 lux. Under these conditions, no ethanol production was observed during the first 14 d in culture.

Ethanol and acetaldehyde were exogenously added to the culture medium immediately after subculturing of unorga-
nized cultures or at d 0 of the process of somatic embryogenesis. Flasks were capped with aluminium caps and sealed with parafilm. The concentration of ethanol and acetaldehyde in control flasks containing only culture medium remained unaltered over the whole experimental period.

The effect of the ADH1 inhibitor on cell growth and somatic embryogenesis was tested. No toxic effects were observed if 4-MP was used at 5 to 10 mM for unorganized cultures and 0.5 to 1 mM for embryogenic cultures.

The pH of the culture medium was monitored and no significant changes were observed following feeding with ethanol, acetaldehyde, or in presence of 4-MP.

Analytical Procedures

Samples of culture media were taken from the cultures and immediately assayed for acetaldehyde using the dimedone reagent as described by Donaldson et al. (6) with minor modifications. In brief, the sample (20–200 μL) was incubated at 100°C for 1 h with 2 mL dimedone reagent (0.3 g 5,5-dimethyl-1,3-cycloexanedione, 2.5 g ammonium acetate, 0.4 mL acetic acid made up to 100 mL water) and 2 mL potassium-phosphate buffer (50 mM [pH 7]). At the end of the incubation at 100°C, fluorescence was measured at 460 nm when excited at 345 nm using a fluorescence spectrophotometer. The amount of acetaldehyde present in the sample was evaluated using a calibration curve. No quenching was observed in the analysis of acetaldehyde content in the culture medium. The results obtained were consistent with those obtained using an enzymatic assay method (Bohringer Mannheim).

Ethanol was assayed by using the method of Bern and Gutman (4).

RESULTS

Influence of Ethanol and Acetaldehyde on Carrot Cell Growth

The influence of exogenously added acetaldehyde on carrot cell growth was tested. The results (Fig. 1) indicate a rapid disappearance of acetaldehyde from the culture medium, mainly due to ADH-catalyzed reduction to ethanol (not shown).

The reduced cell growth observed when acetaldehyde was present in the culture medium seems to be related to the length of cell exposure time. An almost constant 40% growth inhibition was obtained by applying acetaldehyde concentrations ranging from 1 to 5 mM; at all these concentrations the acetaldehyde was rapidly (24 h) depleted from medium. When higher concentrations were used, the acetaldehyde was not completely removed, leading to a longer exposure of cells to the metabolite with the consequence of stronger growth inhibition (60–90%).

To verify whether the presence of a constant amount of acetaldehyde, in absence of ethanol, was able to induce a reduction of cell growth comparable to that induced by the presence of ethanol, acetaldehyde concentration in the me-

![Figure 1. Influence of acetaldehyde on growth of carrot cells. A, Acetaldehyde concentration in the cell culture medium following acetaldehyde treatment; B, effect of acetaldehyde on carrot cell growth. After 168 h cells were filtered and weighed. Data are expressed as percentage of the fresh weight of control cells.](image)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Acetaldehyde Concentration (Mean in the period 1–10 d)</th>
<th>Ethanol Concentration (Mean in the period 1–10 d)</th>
<th>Cell Growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>0.5 ± 0.2</td>
<td>100</td>
</tr>
<tr>
<td>Ethanol 40 mM</td>
<td>0.96 ± 0.09</td>
<td>40 ± 2</td>
<td>43</td>
</tr>
<tr>
<td>Acetaldehyde 1 mM</td>
<td>1.05 ± 0.60</td>
<td>2.1 ± 0.96</td>
<td>37</td>
</tr>
</tbody>
</table>

1 Abbreviations: ADH, alcohol-dehydrogenase; 4-MP, 4-methylpyrazole.
In the medium, acetaldehyde concentration was maintained constant at 1 mM by checking and adjusting acetaldehyde concentration every 12 h.

The results, reported in Table 1, indicate that a relatively low level of acetaldehyde (1 mM), if present for the whole period of cell growth, was able to induce the same toxic effects as 40 mM ethanol (when 40 mM ethanol was added to the medium, acetaldehyde reached 1 mM concentration).

Ethanol-fed carrot cells produce acetaldehyde, which is released in the medium. Twenty-four hours after adding ethanol to the culture medium, acetaldehyde concentration in the medium did not show any further variation but remained almost constant for the whole experimental period (Fig. 2). Carrot cells fed with ethanol were therefore exposed to acetaldehyde at a quite constant concentration dependent on the amount of ethanol present in the culture medium (Fig. 2).

The presence in the medium of 4-MP, an inhibitor of ADH (the enzyme that catalyzes the interconversion ethanol ↔ acetaldehyde), almost completely inhibited acetaldehyde production following ethanol feeding to the cells, as reported in Figure 2.

Therefore, the presence of 4-MP in the culture medium revealed whether ethanol was toxic per se or if the toxicity was due to its oxidation to acetaldehyde. The results, reported in Figure 3, indicate that in presence of 4-MP the toxicity of ethanol was strongly reduced.

The relation between unorganized cell growth and the concentration of acetaldehyde present in the culture medium following feeding with different amounts of ethanol is reported in Figure 4. The linear regression line indicates a correlation between acetaldehyde concentration and cell growth regardless of the concentration of ethanol present in the culture medium.

**Influence of Ethanol and Acetaldehyde on Carrot Somatic Embryogenesis**

As previously reported (16), somatic embryogenesis is more sensitive to the presence of ethanol than is unorganized cell growth.

If ethanol was present in the embryogenic culture medium, acetaldehyde was produced and released in the culture medium (Fig. 5). As for unorganized cultures, the concentration of acetaldehyde present in the culture medium increased during the first 24 h after feeding with ethanol to reach a concentration dependent on the amount of ethanol added to the medium. This concentration remained almost constant during the period 1 to 14 d after the beginning of the experi-
ment (Fig. 5). 4-MP succeeded in inhibiting ethanol oxidation to acetaldehyde even when present at concentrations 10 times lower than those used in unorganized cell cultures: this is probably due to the lower cell density needed for the embryogenic process (concentrations of 4-MP higher than 2 mM are toxic in embryogenic cultures). Figure 6 reports the effects of ethanol on carrot somatic embryogenesis in presence or absence of 4-MP.

A quite normal embryogenesis was obtained even in the presence of ethanol 20 mM if 4-MP was also present in the culture medium.

Figure 6. Influence of ethanol on carrot somatic embryogenesis. Embryogenesis occurred in the absence or presence of the ADH inactivator 4-MP. Embryogenesis was evaluated by counting, under a microscope, the embryos produced (all three stages: globular, heart, and torpedo). Bars indicate ± se (n = 3).

Figure 7 reports the correlation between the concentration of acetaldehyde in the culture medium and the inhibition of somatic embryogenesis. Again, as for carrot cell growth, a linear regression line can be plotted, indicating a correlation between acetaldehyde and embryogenesis regardless of the concentration of ethanol present in the medium.

If acetaldehyde was exogenously added to the culture medium (Table II) some toxic effects were observed, but the embryos produced were normal when compared with those obtained in cultures containing ethanol. This fact is probably due to the brief exposure period of the embryogenic cells to acetaldehyde, since it disappeared from the culture medium (mainly through reduction to ethanol) within 48 h. The presence of 4-MP, which retards acetaldehyde metabolism, allowed one to observe more severe effects of acetaldehyde on somatic embryogenesis (Table II). Nevertheless, the presence of 4-MP failed to completely inhibit acetaldehyde reduction to ethanol; in consequence, no acetaldehyde was detected in the culture medium 3 d after treatment.

The effects observed (Table II) indicate that the presence of acetaldehyde in the first 3 d of embryogenesis inhibited by more than 50% the production of somatic embryos. Nevertheless, the embryos produced in these conditions are quite normal when compared with those produced in the continuous presence of acetaldehyde when ethanol is added to the culture medium.

DISCUSSION

Whether the production of ethanol in plants subjected to anoxia can be harmful has been under debate for a long time. In 1978, Crawford (5) suggested a biochemical basis for the different behavior of plants in anoxia postulating that sensi-
tivity of plants to anoxia originates from the accumulation of fermentation products, mainly ethanol.

Later, several authors reported results in contrast with this theory, since ethanol seems to play only a minor role in causing anoxia injuries (1–3, 10, 17).

Nonetheless, we have shown that, although exogenous ethanol is not harmful on entire seedlings or seeds, it can be toxic at very low concentrations on single tissues or cells (15). Among these, carrot cell culture seems to be a good system to investigate ethanol toxicity on plants, since low amounts of ethanol in the culture medium can strongly interfere with carrot cell growth and somatic embryogenesis (16).

The data presented in this paper emphasize the role of acetaldehyde in ethanol toxicity. Our results clearly indicate that the effects of exogenously added ethanol on carrot cell cultures cannot be ascribed to the presence of ethanol per se but to its metabolism to acetaldehyde.

This view is supported by three lines of evidence. (a) Acetaldehyde continuously maintained at 1 mM concentration (which is the level of aldehyde produced if 40 mM ethanol is added to the medium) caused the same toxic symptoms as ethanol 40 mM. Under these conditions a harmless amount of ethanol was accumulated (about 2 mM). (b) If acetaldehyde production is prevented by adding 4-MP to the culture medium, no toxic effects can be observed even in the presence of very high ethanol concentrations (80 mM). (c) The presence of 4-MP in the embryogenic medium enhances acetaldehyde toxicity by reducing its rate of metabolization.

These results indicate a possible role of acetaldehyde during anoxia. The reports published up to now on acetaldehyde production in plants (11, 12, 14, 18) indicate a different behavior of plant species in terms of acetaldehyde production under anoxia, regardless of the level of ethanol produced.

The mechanism of acetaldehyde toxicity is still unknown, but it has been reported (9) that acetaldehyde can bind to proteins and inactivate enzymes (13). Work is in progress to verify if acetaldehyde-protein adducts occur in acetaldehyde- and ethanol-treated carrot cells and to assess the role of acetaldehyde in anoxia induced injuries.

LITERATURE CITED