Ethanol metabolism in suspension cultured carrot cells

P. Perata and A. Alpi


Ethanol production in plant tissues deprived of oxygen is a well known process. Nevertheless, little information is available on the toxic effects of ethanol on plant cells and tissues, or on the possible role of acetaldehyde, the first oxidative product of ethanol, in inducing toxic effects in plants. Data on the metabolism of ethanol in suspension cultured cells of carrot (Daucus carota L. cv. S. Valery, cell line T22), a system highly sensitive to the presence of ethanol in the culture medium, indicate that carrot cells oxidize only small amounts of ethanol to CO2. Instead, they convert ethanol mainly to acetaldehyde, which accumulates in the culture medium. This suggests a possible role of acetaldehyde in causing ethanol-induced injury to carrot cells.

Key words – Acetaldehyde, anoxia, carrot, Daucus carota, ethanol, in vitro culture, metabolism, Umbelliferac.

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Introduction

It is still unclear whether the production of ethanol is harmful to plant tissues. Several authors have claimed that ethanol has effects other than those due to anoxic conditions; therefore, injury due to anaerobic environments cannot be ascribed to ethanol per se (Jackson et al. 1982, Alpi et al. 1985).

Nevertheless, some systems show high sensitivity to the relatively low ethanol concentrations (10–20 mM) commonly found in plant tissues kept in anoxic conditions (Alpi and Beevers 1983, Perata et al. 1986, 1988). In particular, growth and somatic embryogenesis of carrot cells in vitro seemed highly sensitive to the presence of ethanol in the culture media, and were strongly inhibited by the above-mentioned ethanol concentrations. It is still obscure, however, whether the observed toxicity is due to the presence of ethanol per se or to its metabolism to a more toxic compound.

The first step in ethanol metabolism in plant tissues is its oxidation to acetaldehyde; this oxidative step is considered to be mediated almost exclusively by alcohol dehydrogenase (Cossins 1978). The fate of acetaldehyde depends on the nature of the intermediary metabolism of the different tissues (Cossins 1978), but the presence of an aldehyde dehydrogenase in several tissues suggests its oxidation to acetic acid.

Data on acetaldehyde production in plant tissues are scarce (Cossins 1978, Kimmerer and MacDonald 1987) as well as data on its presence in plant cell cultures (Des S. Thomas and Murashige 1979, Adkins et al. 1990, Righetti et al. 1990). In the present paper we describe some aspects of ethanol metabolism in suspension cultured carrot cells. Our aim was to investigate whether exogenously supplied ethanol was metabolized by carrot cells, and if the presumably more toxic compound acetaldehyde accumulates during this process.

Abbreviations – PCV, packed cell volume; ADH, alcohol-dehydrogenase; EtOH, ethanol; AcCHO, acetaldehyde; 4-MP, 4-methylpyrazole.

Materials and methods

Plant material and culture conditions

Carrot cells (Daucus carota L. cv. St. Valery, cell line T22) were grown in suspension culture in the B5 medium of Gamborg et al. (1968), purchased as powder (Flow laboratories, Irvine, Ayshire, UK). The growth medium was supplemented with 2.26 mM 2,4-dichlo-
rophenoxycetic acid (2,4-D; BDH, Poole, Dorset, UK) and 1.11 mM 6-benzylaminopurine (6-BAP; Sigma, St. Louis, MO, USA).

Culture conditions strongly influence the time at which ethanol production takes place, as well as the amount of ethanol produced (data not shown). In order to minimize endogenous ethanol production, so that only exogenously added ethanol was present in the culture medium, cell culture conditions were slightly modified from those previously described (Perata et al. 1988). Initial cell density was reduced from 1 ml to 0.5 ml PCV, and the speed of the rotary shaker was increased from 80 to 120 rpm. Using these conditions, no ethanol is found in the medium during the first 20 days in culture.

Subculturing was performed every 20 days by inoculating 0.5 ml of packed cells (after centrifugation for 5 min at 200 g) into 25 ml fresh medium. Cultures were kept in flasks at 25°C on rotary shakers (120 rpm) under fluorescent light (Philips TL 40 W/33 RS fluorescent tubes, irradiance = 4 W m⁻²).

Analytical procedures

Samples of culture media were taken at the times indicated and immediately assayed for both ethanol and acetaldehyde.

Ethanol was determined by the method of Bernt and Gutman (1974). Acetaldehyde was assayed according to Donaldson et al. (1985), with minor modifications. The sample (20–100 µl) was incubated at 100°C for 1 h with 2 ml dimedone reagent (0.3 g 5,5-dimethyl-1,3-cyclohexanedione, 2.5 g ammonium acetate, 0.4 ml acetic acid made up to 100 ml with water) and 2 ml potassium phosphate buffer (50 mM; pH 7). At the end of the incubation period, fluorescence was measured at 460 nm with excitation at 345 nm, using a fluorescence spectrophotometer. The fluorescence in the sample was compared to that of known amounts of acetaldehyde (50–400 nmol) developed with dimedone reagent. The reliability of this procedure was carefully checked, and no quenching phenomena were observed in the analysis of acetaldehyde content in culture media. The absence of aldehydes other than acetaldehyde in the culture medium of control and ethanol treated cells was verified by TLC of 2,4-dinitrophenylhydrazone derivatives of carbonyl compounds. Samples (50–100 µl) were extracted for carbonyl compounds (using 500 µl of a saturated solution of 2,4-dinitrophenylhydrazine in 1 M sulphuric acid). After centrifuging (10 min at 13000 g), the pellet was resuspended in a few µl of methanol, and aldehydes and/or ketones possibly present were separated as 2,4-dinitrophenylhydrazones on silica-coated TLC plates (developing solvent hexane: dioxane 90:10 v:v). The following standard aldehydes and ketone were successfully separated using this method: formaldehyde, acetaldehyde, acetone, propanal, butanal, isobutanal, 2-butenal, pentanal, iso-pentanal, hexanal, 2-
nonenal. The 2,4-dinitrophenylhydrazone derivative from carrot culture medium and cell extracts ran to a position identical to that of the derivative of authentic acetaldehyde; no other spots were detected on the TLC plate. Moreover, if cultures of carrot cells were fed 1-[¹⁴C]-ethanol, the 2,4-dinitrophenylhydrazone TLC spot cochromatographing with the acetaldehyde derivative was radioactive.

The reliability of quantitative results obtained using the dimedone procedure was further checked and confirmed by comparison with estimates done using the acetaldehyde enzymatic assay kit from Boehringer Mannheim.

Alcohol dehydrogenase was assayed as previously described (Perata et al. 1988), recording NADH production.

CO₂ evolution was evaluated after feeding cells with 1-[¹⁴C]-ethanol. Flasks were sealed with serum stoppers and, at the times indicated, flushed with sterile air. CO₂ was collected for 1 h in a solution of KOH (14% w/v). One ml of KOH (supplemented with 2 ml ethanol) was taken to dryness to eliminate all the volatile radioactive contaminants present (mainly ethanol and acetaldehyde), resuspended in 2 ml water and counted by liquid scintillation using Opti-Fluor solution from Packard.

Results

Acetaldehyde production started within a few hours after adding ethanol to the culture medium (Fig. 1). After ca 24 h, the increase in acetaldehyde concentration stopped, and then it remained constant or slightly decreasing during the whole period of active cell growth. Cell growth was considerably inhibited by the presence of ethanol, as previously reported (Perata et al. 1988).

Acetaldehyde supplied to the culture medium rapidly disappeared (Fig. 2). At the same time ethanol concentration increased with a time course mirroring that of acetaldehyde disappearance and indicating a likely conversion of acetaldehyde to ethanol. Cell growth in acetaldehyde treated cells was reduced by 40%, regardless of the concentration of acetaldehyde present in the medium (Tab. 1); this is probably due to the short period (6–12 h) of exposition of the cells to the rapidly converted aldehyde.

No alcohol dehydrogenase activity was detected in the culture medium of control or ethanol-acetaldehyde treated cells. Therefore, it was assumed that ethanol and acetaldehyde were metabolized inside the cells. In a previous investigation (Alpi and Beevers 1983) ethanol movements in plant material were shown to be due to diffusion, so that it is unlikely that the rate of uptake and/or exit of ethanol could affect its turn-over.

When acetaldehyde was added to the medium of cells growing in the presence of ethanol (this medium already contained a definite amount of acetaldehyde), the added aldehyde rapidly disappeared until the preexist-
Fig. 1. Ethanol metabolism in suspension cultures of ethanol-fed carrot cells. Carrot cells, in 25 ml fresh medium, were fed with different amounts of ethanol, and both ethanol and acetaldehyde concentrations in the culture medium were monitored at the times indicated. Bars indicate ± se (n = 4 flasks).

levels due to the presence of ethanol were restored (Fig. 3). Cell metabolism accounted for acetaldehyde disappearance, since the concentration of acetaldehyde in control flasks containing only culture medium remained unaltered over the whole experimental period.

Although cytosolic alcohol-dehydrogenase is considered to play a major role in converting ethanol to acetaldehyde (Donaldson et al. 1985), also microsomal CytP450 from rat liver and peroxisomal catalase appear to be able to oxidize ethanol (Oshino et al. 1975, Lieber et al. 1978). In order to check whether a non-ADH pathway was present in carrot cells, we tested their ability to metabolize ethanol and acetaldehyde when grown in the presence of the strong ADH inhibitor, 4-methylpyrazole.

Preliminary experiments indicated that 4-MP completely inhibited carrot ADH activity in vitro if the inhibitor was present at a concentration higher than 1 mM; and that ethanol oxidation to acetaldehyde was almost completely inhibited (Fig. 4), its oxidation being prevented for a long period of time (more than 14 days, data not shown). However, concentrations of 4-MP up to 10 mM had no toxic effects on growth of carrot cells (data not shown). On the other hand, acetaldehyde reduction to ethanol was only slowed down by the presence of the ADH inhibitor but the aldehyde was completely reduced to ethanol within 48 h (Fig. 4). These results indicate that ADH is responsible for ethanol oxidation to acetaldehyde in carrot cells. No significant differences in ADH activity were found between control

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Fresh weight (mg)</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1930 ± 190</td>
<td>100</td>
</tr>
<tr>
<td>Acetaldehyde 1 mM</td>
<td>1250 ± 100</td>
<td>65</td>
</tr>
<tr>
<td>Acetaldehyde 2 mM</td>
<td>1160 ± 80</td>
<td>60</td>
</tr>
<tr>
<td>Acetaldehyde 4 mM</td>
<td>1120 ± 70</td>
<td>58</td>
</tr>
</tbody>
</table>

Fig. 2. Acetaldehyde metabolism in suspension cultures of acetaldehyde-fed carrot cells. Feeding with acetaldehyde, otherwise as for Fig. 1.

Fig. 3. Acetaldehyde metabolism in suspension cultures of carrot cells growing in the presence of ethanol (40 mM). Acetaldehyde was added to the culture medium 48 h after the ethanol. Bars indicate variation width (n = 2 flasks).

Tab. 1. Acetaldehyde toxicity in suspension cultures of carrot cells. Data (± se, n = 3) taken after 14 days in culture.
trols and carrot cells treated with ethanol or acetaldehyde (data not shown). Interestingly, the presence of the ADH inhibitor 4-MP almost completely abolished the toxic effects of exogenously added ethanol and enhanced the toxicity of exogenously added acetaldehyde (Tab. 2).

Carrot cells are able to metabolize small amounts of exogenously supplied \(^{14}\)C-ethanol (Tab. 3). After 48 h, however, only 0.3% to 0.6% of the ethanol supplied was metabolized to CO\(_2\), while a 6 to 9 times higher amount was oxidized to acetaldehyde. 4-Methylpyrazole lowered the level of acetaldehyde but inhibited the production of the labeled CO\(_2\) only slightly. This could indicate that the concentration of acetaldehyde is not a limiting factor for its own oxidation.

**Discussion**

Although the ability of plants to oxidize ethanol has been demonstrated in a wide number of species (Cossins 1978), it has been controversial whether carrot root tissue was able to metabolize ethanol. Feeding carrot root slices with \(^{14}\)C-ethanol, Lowe and James (1960) reported that the release of \(^{14}\)CO\(_2\) was insignificant, but Cossins and Beevers (1963) indicated that, if carrot tissue was fed with micromolar amounts of \(^{14}\)C-ethanol at high specific activity, \(^{14}\)CO\(_2\) evolution could be observed.

Our results show that carrot cells cultured in vitro do not metabolize large amounts of ethanol. In fact only a low percentage of the ethanol fed to the cells is converted to CO\(_2\) (Tab. 3), while a larger amount is oxidized to acetaldehyde. CO\(_2\) evolution from ethanol depends on the concentration of ethanol supplied and, apparently, acetaldehyde concentration has negligible effects on this process (Tab. 3). This phenomenon is difficult to explain, but the possibility of a non-ADH pathway accounting for the small amount \(^{14}\)CO\(_2\) produced cannot be excluded, since the presence of the ADH-inhibitor 4-MP, while strongly reducing the amount of acetaldehyde produced, had only minor effects on \(^{14}\)CO\(_2\) production (Tab. 3). Monk et al. (1987) hypothesized a role for catalase in the oxidation of ethanol, and Donaldson et al. (1985), working with castor bean endosperm, indicated the possibility that a small percentage of ethanol can be oxidized in glyoxyxomes.

Cytosolic alcohol-dehydrogenase seems to be responsible for acetaldehyde production in suspension cultured carrot cells. In the presence of the ADH inhibitor

**Tab. 2. Influence of the ADH inhibitor 4-methylpyrazole on ethanol and acetaldehyde toxicity. Data (± se, n = 3) were taken after 14 days in culture.**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Fresh weight (mg)</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1870 ± 170</td>
<td>100</td>
</tr>
<tr>
<td>Acetaldehyde 4 mM</td>
<td>1030 ± 100</td>
<td>55</td>
</tr>
<tr>
<td>Ethanol 40 mM</td>
<td>370 ± 50</td>
<td>20</td>
</tr>
<tr>
<td>Control + 4-MP 5 mM</td>
<td>1800 ± 170</td>
<td>96</td>
</tr>
<tr>
<td>Acetaldehyde 4 mM + 4-MP 5 mM</td>
<td>750 ± 70</td>
<td>40</td>
</tr>
<tr>
<td>Ethanol 40 mM + 4-MP 5 mM</td>
<td>1700 ± 180</td>
<td>91</td>
</tr>
</tbody>
</table>

**Tab. 3. CO\(_2\) evolution from carrot cells fed with 1-[\(^{14}\)C]-ethanol. Data (± se, n = 3) are expressed in \(\mu\)mol per 25 ml of culture medium.**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>24 h</th>
<th>48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AcCHO</td>
<td>CO(_2)</td>
</tr>
<tr>
<td>EthOH 10 mM</td>
<td>11 ± 1.1</td>
<td>0.8 ± 0.2</td>
</tr>
<tr>
<td>EthOH 20 mM</td>
<td>16 ± 1.5</td>
<td>1.2 ± 0.4</td>
</tr>
<tr>
<td>EthOH 40 mM</td>
<td>25 ± 2.4</td>
<td>1.5 ± 0.3</td>
</tr>
<tr>
<td>EthOH 40 mM + 4-MP 5 mM</td>
<td>4 ± 0.6</td>
<td>1.1 ± 0.4</td>
</tr>
</tbody>
</table>

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4-MP no acetaldehyde production could be observed after feeding ethanol to the cells (Fig. 4D). The failure of 4-MP to inhibit ethanol production from acetaldehyde (Fig. 4B) is consistent with the results reported by Small et al. (1989), who failed to inhibit ethanol production in anoxic Erythrina caffra seedlings using 4-MP treatments. These authors were unable to explain the inefficiency of 4-MP in suppressing alcoholic fermentation, and hypothesized that uptake of the inhibitor could be inefficient or it could be metabolized to some other compound. It could be suggested that the lower $K_m$ of ADH for acetaldehyde, when compared with the $K_m$ for ethanol (Leblova 1978), may account for the lower efficiency of 4-MP in inhibiting acetaldehyde reduction.

The concentration of acetaldehyde increased during the first 24 h from the beginning of the ethanol feeding experiments but then remained constant for the whole active growth period (Fig. 1), while exogenously supplied acetaldehyde was rapidly reduced to ethanol (Fig. 2). No inhibition effects on acetaldehyde reduction were observed if the medium also contained ethanol (Fig. 3), but under these conditions only the amount of acetaldehyde exceeding the concentration due to ethanol oxidation was reduced.

The following considerations can therefore be outlined. When acetaldehyde was supplied to the cells, it was rapidly reduced to ethanol by ADH. If ethanol was fed to the cells, ADH oxidized it to acetaldehyde, and this process went on until the concentration of acetaldehyde reached an equilibrium value, where the speed of ethanol oxidation was equal to that of acetaldehyde reduction, and the concentration of both ethanol and acetaldehyde did not show any further changes.

The $K_m$ of ADH is higher for ethanol oxidation (order of magnitude 10 mM) than that for acetaldehyde reduction (order of magnitude 1 mM) (Leblova 1978). Acetaldehyde accumulation in the culture medium of ethanol treated cells seems, therefore, to be due to the different $K_m$ of ADH for ethanol and acetaldehyde.

Acetaldehyde was not only produced after ethanol feeding but also when carrot cells were cultured at high cell density and with shaking at low speed (data not shown). In these conditions, carrot cells produced ethanol (Perata et al. 1988) and acetaldehyde, which were then metabolized at the end of the stationary growth phase. Further attempts to investigate ethanol metabolism in senescent carrot cells were unsuccessful, due to rapid cell death and lysis.

It can be concluded that exogenously added ethanol is converted to acetaldehyde, which is only slightly oxidized further. The cells are therefore exposed to the presence of the aldehyde for the whole period of growth.

Although it has already been established that ethanol has inhibitory effects on growth of carrot cells (Perata et al. 1988), no data have so far been reported on the effects of acetaldehyde on plant tissues, even though acetaldehyde production has been found in a wide variety of plant species (Kimmerer and MacDonald 1987). However, acetaldehyde may be the main cause of alcoholic liver injuries in animals (Hasumura et al. 1976, von Wartburg and Buhler 1984). The results reported here also indicate a possible role of acetaldehyde in causing ethanol injuries. In particular, results reported in Tab. 2 indicate that in the presence of the ADH inhibitor 4-MP almost no toxic effects can be observed in ethanol treated carrot cell cultures. This indicates that ethanol is likely to be non-toxic in itself, and that the toxicity could derive from a product of its metabolism, probably acetaldehyde, which accumulates in the culture medium of ethanol treated carrot cell cultures. Moreover, the ADH inhibitor 4-MP enhances the toxicity of acetaldehyde (Tab. 2), probably by reducing the rate of its conversion to ethanol (Fig. 4). Work is in progress to elucidate further the role of acetaldehyde on ethanol-induced injury on carrot cells as well as on the ability of senescent carrot cells to metabolize ethanol at high efficiency.

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References


