Polyamines and somatic embryogenesis in carrot. I. The effects of difluoromethylornithine and difluoromethylarginine

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POLYAMINES AND SOMATIC EMBRYOGENESIS IN CARROT. I. THE EFFECTS OF DIFLUOROMETHYLORNITHINE AND DIFLUOROMETHYLARGININE*

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2,4-Dichlorophenoxyacetic acid (2,4-D) is the most commonly used and a very effective inhibitor of somatic embryogenesis in carrot. This growth regulator not only suppresses differentiation of cultured cells but it can also cause a reversion of the developing embryos to undifferentiated callus. Data presented here show that the addition of 1—10 mM DFMO (difluoromethylornithine) to the medium allowed the normal development of somatic embryos to continue even in the presence of inhibitory concentrations of 2,4-D. DFMO caused a significant increase in ADC activity, an increased accumulation of polyamines in the cells, and inhibited the accumulation of ethylene in cell cultures both in the presence or the absence of 2,4-D. Difluoromethylarginine (DFMA) at 0.1—1.0 mM concentration completely inhibited embryogenesis even in the absence of 2,4-D. DFMA also inhibited ADC activity and caused a reduction in the cellular polyamine levels. ODC activity was detected only when fully mature somatic embryos appeared in the cultures.

It is suggested that auxin-induced ethylene biosynthesis plays an important role in the development of somatic embryos in carrot. The promotion of polyamine biosynthesis (by DFMO in the present case) may cause a reduction in the cellular pools of S-adenosylmethionine, which in turn may cause a reduction in ethylene biosynthesis, thus allowing embryogenesis to occur in the presence of an auxin.

Key words: arginine decarboxylase; carrot (Daucus carota); ornithine decarboxylase; difluoromethylarginine; difluoromethylornithine; ethylene; polyamines; somatic embryogenesis

Introduction

The elucidation of biochemical changes in cells during embryogenesis may present the key to experimental control of regeneration in plant cells cultures. Carrot (Daucus carota L.) cell suspension cultures provide an excellent model system for such investigations [1,2]. Somatic embryogenesis in carrot can be regulated by 2,4-dichlorophenoxyacetic acid (2,4-D), a synthetic auxin. Embryo development is initiated upon removal of 2,4-D from the medium [1,3,4]. Although cell lines which do not differentiate in the absence of 2,4-D have been isolated [5,6], similar cell lines which undergo normal embryogenesis in the presence of 2,4-D are not available. Furthermore, it is known that the addition of 2,4-D to the medium even at late stages of embryogenic development causes rapid dedifferentiation of developing embryos into callus.

There are many changes in the primary and secondary metabolism of carrot cell cultures...
during embryogenesis, including significant changes in the levels of polyamines [7–9]. The polyamines, putrescine, spermidine and spermine, are ubiquitous in living systems, and have a variety of physiological functions in plants and animals [10–14]. Previous investigations indicate an important role of polyamines during morphogenesis in plant cell cultures [14–16]. Montague et al. [17] found that polyamine levels increased in carrot cells transferred to embryogenic medium. Fienberg et al. [9] further showed that in a non-embryogenic cell line of carrot, the levels of polyamines remained quite low both in the presence and the absence of 2,4-D.

In animals there is only one pathway for polyamine biosynthesis, and ODC is the enzyme catalyzing the rate limiting reaction [10,13,14]. However, plants have an additional polyamine biosynthetic pathway utilizing ADC as the enzyme catalyzing the rate limiting reaction [12,14]. The relative importance of the two pathways during development and in different tissues is not clear at present.

Montague et al. [17] reported that ADC activity increased concomitantly with an increase in cellular polyamines during embryogenesis in carrot cultures. In contrast, ODC remained low and unchanged, suggesting that ADC may play a more important role during somatic embryogenesis than ODC. Corroborating these findings, Feierer et al. [8] showed that DFMO, a specific irreversible inhibitor of ODC, did not affect embryogenesis in carrot, whereas DFMA, a similar inhibitor of ADC, inhibited the development of somatic embryos. More recently, Mengoli et al. [18] reported increased growth rates and higher putrescine and spermidine levels in carrot cells grown in the presence of DFMO and 2,4-D for several months. The effect of DFMO on the differentiation process was not reported.

The present report provides further insight into the possible role of polyamines in the developmental regulation of somatic embryos in carrot by 2,4-D. Continued normal development of somatic embryos was seen in the presence of 2,4-D if DFMO was present in the medium. DFMO promoted ADC activity as well as polyamine biosynthesis in these cells.

Materials and Methods

Cell culture and growth conditions

The cell cultures of wild carrot (Daucus carota L.) were obtained from Dr. Wetherell, University of Connecticut. Cultures were maintained in B5 medium [19]. 2,4-D was added before autoclaving. Stock solutions of DFMO and DFMA (100 mM) were made in deionized H₂O, the pH adjusted to 5.2, the solutions filter sterilized and then added to the pre-cooled (55°C) medium to obtain desired concentrations.

At the time of transfer, cells from liquid cultures were passed through a 200 µm or 1 mm sieve and washed twice with 2,4-D-free medium by centrifugation at 200 rev./min. Cells were resuspended in fresh medium and pipetted into 50 ml medium using wide bore 10-ml pipets. The stock cultures were generally diluted 1:20 every 15 days, (inoculum approx. 60–100 mg/50 ml). Cultures were maintained under 90 µE s⁻¹ m⁻² of light at 24 ± 1°C on gyratory shakers at 150 rev./min. Solid cultures were maintained in Petri dishes wrapped in cellophane under similar conditions.

Enzyme assays

ODC and ADC were extracted and assayed by modification of the procedure of Branca and Herbst [20]. Cultures were removed from the shaker just before grinding and cells collected on a Whatman No. 1 filter paper in a Buchner funnel. Cells were homogenized in the extraction buffer (2 ml/g fresh wt.) with polyvinylpolypyrrolidone and glass beads in a chilled mortar and pestle. The extraction buffer (referred to as TAB) contained 0.05 M Tris–HCl, pH 8.5 adjusted at 4°C, 0.5 mM pyridoxal-5-phosphate, 0.1 mM Na₂-ethylenediamine-tetraacetate (EDTA), and 5.0 mM dithiothreitol. The homogenate was centrifuged at 20 000 × g for 20 min and the supernatant fluid used for enzyme assays.

ADC and ODC activities were determined by
measuring the amount of $^{14}$CO$_2$ released from L-[U-$^{14}$C]arginine (spec. act. 61 mCi/mmol) or DL-[1-$^{14}$C]arginine (spec. act. 61 mCi/mmol) and L-[1-$^{14}$C]ornithine (spec. act. 61 mCi/mmol), respectively. No difference in the activity of ADC was seen by using either DL-[1-$^{14}$C]- or L-[U-$^{14}$C]arginine. The reaction mixture contained (in a final vol. of 0.5 ml): 0.25 ml TAB, 0.05 ml of 20 mM L-arginine containing 0.1 μCi [$^{14}$C]arginine, and 0.2 ml of centrifuged homogenate. In the case of ODC, 0.05 ml of 20 mM ornithine containing 0.1 μCi of [$^{14}$C]ornithine was used instead of arginine. The blanks contained reaction mixture without the supernatant fraction. The tubes were sealed with rubber caps fitted with plastic center wells containing a 1 cm$^2$ Whatman No. 3 filter paper soaked with 0.05 ml of Tissue Solubilizer (Beckman, B450). The reaction mixture was incubated at 37 °C for 60 min and the reaction terminated by injecting 0.5 ml of 0.2 M H$_2$SO$_4$ through the rubber cap. Following an additional 60 min incubation, the filter papers were placed in scintillation vials with 10 ml of organic scintillation fluid. Vials were kept in the dark for at least 1 h before counting in a LS7000 Beckman Liquid Scintillation Counter. Protein content of the supernatant fraction was determined by the method of Bradford [21]. One unit of enzyme activity is defined as 1 nmol CO$_2$ (mg protein)$^{-1}$ h$^{-1}$.

**Polyamine extraction and quantification**

Polyamines were extracted and quantified by dansylation and fluorimetry [22]. The cells were homogenized in ice-cold 5% perchloric acid (5 ml/g fr. wt.) in a chilled mortar and pestle. The extract was poured into a chilled Corex centrifuge tube, incubated on ice for 1 – 2 h, and centrifuged at 10 000 $\times$ g for 30 min at 4 °C.

Polyamine standards and the extracts (0.2 ml) were pipetted into conical centrifuge tubes containing 30 mg sodium bicarbonate. Dansyl chloride (400 μl) (20 mg/ml in acetone) was added to each tube. Tubes were vortexed briefly, capped and incubated overnight in the dark at 37 °C. Excess dansyl chloride was reacted with 0.1 ml of L-proline (100 mg/ml). The tubes were vortexed and incubated at 37 °C for an additional 60 min. Acetone was evaporated from the solutions under vacuum. One milliliter of toluene (Photrex, Baker) was added to each tube and the mixture vortexed for 60 s. Following centrifugation at 2000 rev./min for 20 min, the toluene layer was used for thin layer chromatography using silica gel plates (Baker, Si 250-PA). The solvent mixture contained cyclohexane: ethylacetate (3 : 2 v/v). After the solvent front had reached 1 cm from the top of the plate, they were removed and sprayed with isopropanol : triethanolamine (4 : 1 v/v) and dried for 5 – 10 min. The plates were then placed in a vacuum oven at 55°C for 60 min in complete darkness. The plates were scanned in a Shimadzu TLC fluorescent scanner at excitation and emission wavelengths of 265 nm and 510 nm, respectively. Concentrations of the unknown samples were calculated from a standard curve of known concentrations (50 – 400 μmol). Spermine estimatations were also done by HPLC of dansylated polyamines using a fluorescence detector [23].

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**Table I.** Summary of the results on the effect of different concentrations of DFMO on the reversal of somatic embryogenesis by 2,4-D in carrot cell cultures. DFMO and 2,4-D were added after 7 days of growth in the 2,4-D-free medium and the observations made after a total of 16 days. Data are representative of 4 experiments.

<table>
<thead>
<tr>
<th>2,4-D (mg/l)</th>
<th>DFMO (mM)</th>
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<tr>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>0</td>
<td>+</td>
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<tr>
<td>0.01</td>
<td>+</td>
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<td>0.05</td>
<td>0</td>
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<tr>
<td>0.10</td>
<td>0</td>
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<td>0.50</td>
<td>0</td>
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<tr>
<td>1.0</td>
<td>0</td>
</tr>
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*Relative differentiation (greening of cell clumps and cotyledon growth) Scale: 0 = no differentiation; + = least amount of differentiation; ++ ++ = Maximum differentiation as observed in control, i.e minus 2,4-D cultures.*
Results

Effects of DFMO and DFMA on embryogenesis

Cells remained undifferentiated in the medium containing 0.1 or 0.5 mg/l 2,4-D. Globular embryos appeared within 7-10 days after transfer to 2,4-D-free medium and mature embryos were abundant by 14-20 days of culture. When 2,4-D was added to cultures after 2, 5 or 10 days of growth in 2,4-D-free medium, normal growth of the differentiating embryos ceased within 2 days and callus proliferation began. The first sign of a 2,4-D effect was the appearance of a rough surface on otherwise smooth embryos. If 1-10 mM DFMO was added along with 2,4-D, normal development of embryos continued although the growth rate was slower. Addition of DFMO to the 2,4-D-free medium had no effect on embryo development or growth. Thus, the dedifferentiating effects of 2,4-D were completely reversed by DFMO.

To further investigate the interaction of DFMO and 2,4-D, different concentrations of 2,4-D ranging from 0.01 to 1 mg/l were added along with 1, 10 or 20 mM DFMO to 7-day-old cultures growing in 2,4-D-free medium (Table I). Embryogenesis was scored after an additional 10-14 days. In all cases where no DFMO was added, 2,4-D suppressed the development of embryos and caused callus formation. One millimolar DFMO was sufficient to overcome the dedifferentiating effects of lower concentrations of 2,4-D (0.01 and 0.05 mg/l); whereas 10-20 mM DFMO was required for normal growth of embryos in the presence of higher concentrations of 2,4-D.

Following the initial results with cultures which contained differentiating embryos, additional experiments were performed to determine if DFMO could counteract the effects of 2,4-D on callus cells grown continuously in the presence of inhibitory concentrations of 2,4-D (0.1-0.5 mg/l). Addition of 1-10 mM DFMO to cultures on the first day of transfer to fresh 2,4-D containing medium also resulted in a reversal of the inhibition of somatic embryogenesis by 2,4-D. However, in these cultures, embryogenesis was slower than in the absence of 2,4-D, and a smaller proportion of cell clumps produced embryos. Globular embryos appeared in large numbers between 10 and 14 days of culture as compared to 7-10 days in 2,4-D free medium. Results were comparable in both the solid and the liquid cultures.

In contrast to the effects of DFMO, 0.1 mM DFMA when added from the first day of culture completely inhibited embryogenesis. Higher concentrations of DFMA (1.0-10 mM) inhibited growth as well as embryogenesis both in the presence or absence of 2,4-D. The effects of DFMA on growth and embryogenesis were reversed by the addition of 1-10 mM putrescine (data not shown).

Effect of DFMO on ODC and ADC activity

The reversal of 2,4-D-induced suppression of embryogenesis by DFMO suggested that a modulation of ODC and/or ADC activities by this compound might influence polyamine levels and hence, differentiation. Activities of both ODC and ADC were assayed at 2-day intervals in cells grown with or without 2,4-D and/or 10 mM DFMO.

Prior to enzyme assays, the procedure for determination of enzyme activities was opti-
mized with respect to substrate concentration, pH and temperature. The pH optimum for both the enzymes was determined to be 8.5 (data not presented). The rate of reaction was linear for up to 90 min at 37°C for both enzymes. The activity of ADC as determined by using [U-14C]arginine was not inhibited by 10 μM phenyl phosphorodiamidate, a potent irreversible inhibitor of urease [23], showing that the release of 14CO2 from arginine was not due to a combined action of arginase and urease [23a]. Furthermore, there was no difference in the observed ADC activities whether [1-14C]- or [U-14C]arginine was used as the substrate. Whereas ADC activity was strongly inhibited in vitro by DFMA and not by DFMO, ODC activity (tested only from mature leaf tissue) was inhibited neither by DFMO nor by DFMA.

When cultures were transferred to fresh media containing 2,4-D, ADC activity increased, peaked at day 2 and then declined to moderate levels during the remainder of the 16-day culture period (Fig. 1). DFMO strongly promoted ADC activity during the first 10 days. A similar effect of DFMO on ADC activity was seen in cells grown in the absence of 2,4-D; the promotory effect being visible only during the first 4 days. Ornithine decarboxylase activity, on the other hand, was very low and remained unchanged during the first 10—12 days of culture in all cases (± 2,4-D, with and without DFMO) (Fig. 2). After this time, there was a sharp increase in ODC activity in cultures that had produced mature, green somatic embryos. The enzyme activity increased 3—10-fold during the next 10 years. In some experiments, as much as a 30-fold increase in ODC was seen at this time. In contrast, non-embryogenic cells showed consistently low ODC activity throughout the entire culture period. In 2,4-D + DFMO cultures, which produced mature green embryos by 20—24 days, ODC activity appeared only around day 20.

**Effect of DFMA on ADC and ODC activities**

In contrast, to DFMO, DFMA (0.1 mM) had an inhibitory effect on the activity of ADC in cells growing both in plus and minus 2,4-D media (Fig. 3). All cultures showed the usual increase in ADC activity at 2 days following the transfer to fresh media. Cultures with DFMA, however, showed only a slight increase of ADC at this time. ADC activity declined in all

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**Fig. 2.** Changes in ODC activity in carrot cell cultures grown in the presence or absence of 0.5 mg/l 2,4-D during a 20-day time course. (○) = minus 2,4-D, (△) = plus 2,4-D, (●) = 2,4-D plus DFMO. All cultures were transferred to fresh medium (containing the same constituents) without dilution after 16 days. Each point is a mean of 4 replicates.

**Fig. 3.** Effects of 0.1 mM DFMA on ADC activity in carrot cell cultures grown in the presence or absence of 0.5 mg/l 2,4-D during a 20-day time course. (○) = plus 2,4-D, (△) = minus 2,4-D, (●) = 2,4-D plus DFMA, (●) = minus 2,4-D plus DFMA. All cultures were transferred to fresh medium (containing the same constituents) without dilution after 16 days. Each point is a mean of 4 replicates.
cultures by day 6, rising again only on transfer to fresh media (day 20). Cells grown in the presence of DFMA without 2,4-D seemed to have recovered from the DFMA effect since they showed an increase in ADC activity on day 14 and a further increase on day 20. These cells also showed somatic embryo development by day 20. No such recovery in ADC was seen in the presence of 2,4-D. ODC activity remained negligible during the first 14 days and was not affected by DFMA in any case (data not shown). DFMA (1–10 mm) caused a severe inhibition of growth as well as of embryogenesis. In addition there was no recovery in ADC activity and there was no embryogenesis in cultures exposed to these levels of DFMA. ADC was almost non-detectable by 4 days of culture and thereafter.

**ADC and ODC activity in mature wild carrot**

During the 20-day time course (Fig. 2), an increase in ODC activity was seen only after the differentiated embryos had developed green cotyledons. In order to test the possibility that ODC in carrot may show an organ-specific distribution, the relative levels of ODC and ADC activities in different parts of mature plants were determined. The plants were harvested from the field in late summer. It was observed that ODC activity was significantly higher than ADC in leaf tissue (Fig. 4). ODC and ADC activities in flowers were not significantly different, whereas in roots, ODC was undetectable. Specific activity of ADC was also lower in the roots as compared with either leaves or flowers. Normal plantlets growing in culture for 2–3 months also showed significant amounts of ODC (data not shown).

**Effect of DFMO and DFMA on cellular polyamines**

In the absence of DFMO, 2,4-D had little effect on putrescine concentrations in the tissue (Fig. 5). Putrescine levels were the highest in all cases 2 days after transfer and declined sharply by day 6 and stayed low thereafter. DFMO significantly promoted cellular putrescine at all times in the presence of 2,4-D and

![Fig. 5. Effect of 10 mM DFMO on cellular levels of putrescine and spermidine in carrot cell cultures grown in the presence or absence of 0.5 mg/l 2,4-D. Polyamines were determined by TLC. Values are means of 3 replicates. Data analyzed using two-way ANOVA and significance tested among the treatments at a given time only.](image)
only on day 2 in the absence of 2,4-D. Spermidine, which was the most abundant of the three polyamines, increased up to day 6 both in the presence and absence of 2,4-D, being significantly higher in the latter on day 2. DFMO, promoted spermidine only in the presence of 2,4-D, the effect being seen on all the days tested.

An accurate quantitation of spermine was difficult by TLC in the cells treated with DFMO because a strong fluorescent band [presumably a metabolite of DFMO, but certainly not DFMO itself (details not presented)] was seen slightly ahead of the spermine band which caused aberrant fluorescence readings. Therefore, the spermine analysis was later performed with HPLC. The HPLC analysis showed much lower spermine concentrations than comparable data from TLC. Regardless of how the analysis was done, it was clear that DFMO significantly promoted spermine levels in the tissue grown either in the absence or presence of 2,4-D (Fig. 6). A maximum of 50% increase in spermine was seen with DFMO on day 10.

The effect of 0.1 mM DFMA on polyamine levels was determined at 3 and 5 days of culture, around the period of maximum inhibition of the ADC activity. As expected, the total polyamine levels in cells grown in the presence of DFMA were significantly lower than in the respective controls. In terms of relative effects, DFMA treatment resulted in lower putrescine and spermidine concentrations, while spermine concentrations were slightly promoted (Fig. 7). In 2,4-D + DFMA cultures, putrescine was not detectable on either day.

**Effects of DFMO on ethylene production**

Ethylene concentrations were measured in flasks containing cultures growing in the presence and the absence of 2,4-D and DFMO. Although there was considerable variation among the results from different experiments with regard to the absolute ethylene concentrations, the trends were always consistent with the data presented in Table II. Accumulation of entylene was significantly lower in the absence of 2,4-D as compared to that in its presence, both on days 3 and 6 of culture. DFMO reduced

![Fig. 6. Effect of 10 mM DFMO on cellular levels of spermine in carrot cell cultures grown in the presence or absence of 0.5 mg/l 2,4-D. The abscissa represents days. The upper half shows spermine determined by TLC and the lower half shows spermine determined by HPLC. Values are means of 3 replicates. TLC data analyzed as in Fig. 5 and HPLC data analyzed by Scheffe’s One-way ANOVA using STATA version 1.5 (Computing Resource Center, Los Angeles, CA)](image)

![Fig. 7. Effect of 0.1 mM DFMA on cellular levels of polyamines in carrot cell cultures grown in the presence or absence of 0.5 mg/l 2,4-D. Polyamines were determined by TLC. Values are means of 2 replicates.](image)
Table II. The effect of DFMO (10 mM) on the accumulation of ethylene in carrot cultures grown for 3 and 6 days in the presence or absence of 2,4-D. Data are means ± S.E. of three replicates. Treatments with similar letters were compared with each other within the column. *Denotes significant differences from the control at $P < 0.05$, **$P < 0.01$. (Data analyzed by Scheffé's One-way ANOVA using STATA version 1.5 (Computing Resources, Inc., Los Angeles, CA).

<table>
<thead>
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<th>Treatment</th>
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<tbody>
<tr>
<td></td>
<td>3 days</td>
</tr>
<tr>
<td>+ 2,4-D</td>
<td>922 ± 33 A*B**</td>
</tr>
<tr>
<td>- 2,4-D</td>
<td>638 ± 41 C<strong>B</strong></td>
</tr>
<tr>
<td>DFMO</td>
<td>517 ± 16 C**</td>
</tr>
<tr>
<td>DFMO + 2,4-D</td>
<td>736 ± 25 A*</td>
</tr>
</tbody>
</table>

ethylene accumulation to the same extent both in the presence and absence of 2,4-D. Although aminooxyacetyl glycine (AVG), an inhibitor of ethylene production, promoted embryogenesis at low concentration (2—5 μm) in the absence of 2,4-D (green embryos appeared by days 10—12, instead of 14—16), it could not induce embryogenesis in the presence of 2,4-D.

Discussion

The most commonly used and effective inhibitor of somatic embryogenesis in carrot cell cultures is 2,4-D [1,4]. This growth regulator not only suppresses differentiation of cultured cells but it can also cause reversion of the developing embryos to undifferentiated callus. This effect is only reversible by removal of 2,4-D from the medium. We have determined that the modulation of polyamine biosynthesis by addition of DFMO to media containing 2,4-D results in almost a complete reversal of the dedifferentiating effect of 2,4-D. This effect does not seem to be related to interference with the uptake of 2,4-D by the cells (data not presented), although functional interaction at the membrane level cannot be ruled out.

In addition to confirming the previous reports indicating that polyamine biosynthesis is important during differentiation of somatic embryos in carrot, our results provide further insight into the possible roles of ODC and ADC in this process. While ODC activity remained undetectable during the first 10—12 days of embryogenesis (development to almost normal embryos), a sharp rise in ODC was seen after that period coinciding with the appearance of mature, green plantlets. Similarly, tissues from carrot plants growing in vitro as well as in the field showed significantly higher ODC activity indicating that ODC in carrot may be restricted to green tissues. ADC, which was the predominant enzyme during the first 2 weeks in culture showed reduced activity in mature embryos.

The most interesting observation, however, is the morphogenetic effect of DFMO, which is a known specific inhibitor of ODC in most plant and all animal tissues [10,13,14]. While having no apparent effect on the extractable ODC activity from either differentiating or non-differentiating cells, DFMO caused an increase in ADC activity in these cells. Moreover, when DFMO was added to the differentiating cultures along with inhibitory concentrations of 2,4-D, it allowed embryogenesis to proceed normally. The DFMO-induced embryogenesis was preceded by increased ADC activity and an increased accumulation of polyamines in the cells. These results are in agreement with the findings of Fallon and Phillips [24], who reported that DFMO, while having no effect or a slight promotion of growth in carrot cell cultures, promoted ADC activity in these cells. No such effect on ODC or ADC was seen by Mengoli et al. [18]. Fallon and Phillips [24] also reported a significant decrease in ADC as well as the polyamine content in the presence of DFMA.

Based on the earlier published work on carrot cell cultures and the data presented here, it is known that: (i) auxin suppresses somatic embryogenesis [1—4]; (ii) ethylene inhibits somatic embryogenesis [25,26]; (iii) DFMO allows somatic embryogenesis to proceed even in the presence of auxin; (iv) increased polyamine biosynthesis (especially spermidine and spermine) accompanies embryogenesis in the
presence of DFMO; and (v) lower levels of ethylene accumulate in the presence of DFMO. In addition, it is also well established that: (i) exogenous auxin stimulates ethylene biosynthesis in many plant tissues [27–29]; and (ii) the pathway for spermidine and spermine biosynthesis shares a common precursor (S-adenosylmethionine) with ethylene biosynthesis [10–12]. Although the exact mechanism of how DFMO overcomes the inhibitory effects of 2,4-D is still not clear, a working hypothesis can be suggested.

The hypothesis has two postulates: (1) Ethylene is an effective suppressor of somatic embryogenesis and its production is promoted by auxin; and (2) the increased biosynthesis of polyamines, especially spermidine and spermine is required for embryogenesis and its promotion (by DFMO in the present case) depletes the cellular pools of S-adenosylmethionine, thus causing a further reduction in ethylene biosynthesis. A close metabolic link between ethylene and polyamines is evident from the fact that their biosynthesis pathways share and probably compete for the same intermediate, i.e. S-adenosylmethionine. It has been reported that inhibition of ACC synthesis stimulates the incorporation of radioactivity from methionine into spermidine and spermine [30]. Likewise it has been shown that the inhibition of polyamine synthesis by D-arginine, DFMA or MGBG, results in a significant increase in ethylene production [31]. An increased synthesis of spermidine was shown to reduce the endogenous concentrations of ACC and also inhibited the conversion of ACC to ethylene [32–34].

Our studies on the effects of exogenously supplied polyamines further show that while putrescine (1–10 mM) has no effect on embryogenesis in the 2,4-D-free medium, both spermidine and spermine inhibit embryo development at these concentrations with spermine being more effective (unpublished data). Although our results are in general agreement with the earlier reports that increased levels of polyamines are essential for embryogenesis to proceed normally, we further believe that it is not merely the presence of "high" polyamine levels in the cells that is important (which could be achieved through exogenous supply of spermidine and spermine), rather it is the increased biosynthesis and probably a fast turnover of spermidine and spermine which is critical. It is only through increased synthesis of these metabolites that SAM can be diverted away from ethylene biosynthesis. In the presence of exogenous spermidine and spermine, in fact, one would expect a reduced polyamine biosynthesis and an increased ethylene biosynthesis.

Two points should, however, be made with regard to the above hypothesis. First, although this hypothesis is quite compatible with most of the published work, it is certainly not a complete explanation for the 2,4-D effects on embryogenesis. Secondly, while AVG and AOA, two of the strong inhibitors of ethylene biosynthesis, promote embryogenesis in the absence of 2,4-D (unpublished results), they are not able to induce embryogenesis in the presence of 2,4-D. A possible explanation for this observation may be that AVG and AOA could directly affect the polyamine biosynthetic enzymes, e.g. ADC and SAM decarboxylase. However, the hypothesis can be experimentally tested because a number of inhibitors for each step in polyamine as well as ethylene biosynthesis are available [10,14]. Direct experimental evidence is needed to strengthen this hypothesis and thus gain further knowledge about the regulation of somatic embryogenesis by auxin and polyamines in carrot cell cultures.

Acknowledgments

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