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Polyamines and cytokinins in celery embryogenic cell cultures

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Abstract

The effect of inhibitors of polyamine biosynthesis on the development of embryogenic cell cultures of celery (*Apium graveolus* L.) was studied. Several developmental stages of somatic embryos were compared for differences in the content and biosynthesis of free polyamines and for cytokinin content. Cyclohexylamine and particularly methylglyoxal *bis*(guanylhydrazone), inhibited both cell division and the organization of polar embryos from globular embryos. Difluoromethylornithine slightly promoted embryo development, especially cell division.

The free putrescine content of globular embryos was 6-fold that of fully differentiated plantlets, and that of spermidine 2-fold. Only a slight increase in the spermine content was found with embryo development. These differences were confirmed by data from polyamine biosynthesis. Incorporation of ^{14}C-arginine into polyamines was slightly higher than that of ^{14}C-ornithine. Over 96% of this incorporation was detected in the putrescine fraction. Incorporation of ^{14}C into putrescine in globular embryos was 3 to 4-fold that in fully-differentiated plantlets. Incorporation into spermidine and spermine was, however, higher in plantlets than in globular embryos.

Cytokinin analysis revealed considerable differences in the biological activity between the developmental stages of embryogenesis. This could be due to endogenous cytokinins and/or BA taken up from the maintenance medium. Cytokinin levels decreased with increased embryo development. Most of the detected cytokinin-like activity co-chromatographed with BA and its metabolites. Some as yet unidentified peaks of activity were recorded in the globular embryos.

The results are considered with respect to the possible participation of polyamines and cytokinins in the development of embryogenic cell cultures of celery. It is suggested that the onset of embryogenesis is characterized by a high content of putrescine and cytokinins, while a decrease in putrescine synthesis and cytokinin content, and an increase in spermidine and spermine content, accompany further embryo development and plantlet formation.

Abbreviations: ADC = arginine decarboxylase; ODC = ornithine decarboxylase; 2,4-D = dichlorophenoxyacetic acid; DFMA = difluoromethylarginine; DFMO = difluoromethylornithine; MGBG = methylglyoxal *bis*(guanylhydrazone); CHA = cyclohexylamine; BA = benzyladenine; BAR = benzyladenine riboside

1. Introduction

Recent investigations showed that active cell division and treatments with all five major groups of plant hormones result in significant changes in polyamine content and biosynthesis in many plant species and in many developmental processes, including cell culture and somatic embryogenesis [2, 19]. The interactions of polyamines with auxins, gibberellins and especially ethylene is well researched. The interrelationships of polyamines with cytokinins and abscisic acid, however, have been studied only occasionally.

Cellular and morphogenetic events during somatic embryogenesis are controlled by an array of culture conditions (medium composition and physical environment), and by genotypic effects [4, 5]. The differential requirement for auxin during the embryo induction and maturation stages is well established [5, 17]. However, the involvement of cytokinins in somatic embryogenesis is less clear, although most nutrient media for embryo induction contain benzyladenine, kinetin or both. The importance of cytokinins at the initial cell division phase of somatic embryogenesis in carrot was established by Fujimura and Komamine [17, 18]. Subsequent studies of somatic embryogenesis in anise cell cultures (7, 8, 9) have shown high levels of zeatin and *iso*-pentenyladenine nucleotides during the initial logarithmic growth of the cultures. This was not the case at later stages, suggesting that cytokinins may have a role in cell division but not in embryo differentiation. A general increase in total cytokinins (as judged by the *Amaranthus* bioassay) was noted in mature embryoids of celery [1]. Two recent studies suggest that somatic embryogenesis in cereals and grasses is inhibited by endogenous cytokinins. Both non-embryogenic leaf regions and callus of *Pennisetum purpureum* contained higher levels of cytokinins than the highly embryogenic material [28]. Non-embryogenic genotypes of orchardgrass contained 3 to 4-fold higher concentrations of zeatin and zeatin metabolites than embryogenic genotypes [31].

Several studies suggest a role for polyamines in carrot somatic embryogenesis, as inferred from changes in polyamine content, the activity of polyamine biosynthetic enzymes, and from the effect of inhibitors of polyamine biosynthesis [6, 11, 12 13, 21, 23, 24, 29]. A requirement for polyamines and ADC activity, in combination with 2,4-D, was established by the use of DFMA, MGBG and CHA [11, 13]. The inhibitory effect of DFMA, but not of DFMO, was correlated with auxin-induced ethylene biosynthesis [29]. The differential role of polyamines during several embryogenic stages in carrot (including the effect of polyamine biosynthesis inhibitors) was reported recently [21]. The involvement of polyamines in somatic embryogenesis, was also studied in eggplant cotyledons [14].

The availability of a highly synchronized and productive somatic embryogenesis system in celery [25, 26, 27] resulted in the study of polyamine involvement in this process. It was previously reported that MGBG (an inhibitor of S-adenosylmethionine decarboxylase and, thus, of spermidine and spermine synthesis) inhibited growth of celery embryogenic clumps and shoot differentiation, whereas addition of spermidine to the culture medium enhanced embryo differentiation and plantlet formation [3]. Changes in polyamine content during somatic embryogenesis were also noted [4].

In this paper we report on the effect of polyamine biosynthesis inhibitors on cell growth and embryo development of celery cultures. Data of simultaneous changes in polyamine content and biosynthesis, and in cytokinin content (both endogenous and applied) in defined size fractions of embryos are presented.

2. Materials and methods

2.1 Cell and embryo cultures

Embryogenic cell suspension cultures of celery (*Apium graveolens* L. var. SB 12) were established from secondary callus of line B (i.e., SCB) as described previously [25, 26]. Cell suspension cultures were maintained routinely by subculturing every 2 weeks in a "maintenance medium" (MS basal medium supplemented with 500 mg l^{-1} casein hydrolysate, 30 g l^{-1} sucrose, 4.6 μM 2,4-D, 0.88 μM BA, and 40 g l^{-1} mannitol). For embryo development and plantlet regeneration, suspension cultures were transferred to a "regeneration medium" (half-strength MS basal medium supplemented with 500 mg l^{-1} casein hydrolysate, 15 g l^{-1} sucrose and 1.15 μM kinetin). All suspension cultures were maintained on a rotary shaker

(105 rpm) at 26 ± 1°C and a 16 h photoperiod of cool white fluorescent light (50–60 μE). After 2 to 3 weeks in suspension culture, small plantlets were transferred to filter paper supports in vials with liquid regeneration medium. This resulted in fully-developed plantlets [26, 27].

Suspension cultures from maintenance or from regeneration media were used either directly or following sieving through metal mesh screens of 200–2400 μm size, resulting in defined size fractions of somatic embryos [26]. Shoots of plantlets which developed on filter paper supports were also used. All details are indicated in the legends to Figures and Tables. Polyamines and inhibitors were added directly to maintenance or regeneration media. Growth of cultures was determined either using cell and embryo counting, packed cell volume, or fresh wt.

2.2 Polyamine analysis

Cultures were washed with distilled water using delicate vacuum filtration, surface-dried with filter paper, and their fresh wt determined. Free polyamines were extracted in 5% perchloric acid, dansylated, separated on TLC plates (Merck, precoated with silica gel 60), and then quantitated fluorimetrically as described previously [16, 30]. Routine TLC separation of free polyamines was verified by HPLC separation following standard benzoylation of extracts.

Polyamine biosynthesis was determined by incorporation of labelled precursors as described previously [16]. Briefly, cultures were incubated for 5 h with U-^{14}C-arginine (NEN, 320.5 mCi/mmol), or U-^{14}C-ornithine (NEN, 283 mCi/mmol), or 1,4-^{14}C-putrescine (NEN, 90.4 mCi/mmol). Radiolabelled precursors were included in the maintenance or regeneration media at a rate of ca. 2 μCi 10 ml^{-1} suspension, equivalent to ca. 1 g fresh wt. Following incubation, cultures were washed 3 times with cold medium using vacuum filtration, delicately dried with filter paper, weighed and extracted. The extracts were separated on TLC plates as described above. Radioactivity of the various polyamine spots was measured in soluene (Packard), using a liquid scintillation counter.

Experiments were performed twice (Fig. 3, Table 1) or 3 times (Figures 1,2 and Table 1), 2–3 replicates each (as mentioned in the legends). Results of a typical experiment are presented.

2.3 Cytokinin analysis

Three size fractions of cell cultures (500–1400 μm, 1400–2000 μm and > 2000 μm), cultured in regeneration medium, were washed extensively with distilled water using delicate vacuum filtration, surface-dried with filter paper, weighed, placed immediately in liquid N_2, and then freeze-dried. The resulting dry plant material was used for cytokinin analysis. The equivalent of 10 g fresh wt of each sample was placed in 50 ml 80% re-distilled ethanol and extracted by agitation for 24h. After filtration, the extracts were dried under vacuum. Each extract was subsequently resuspended in 50 ml 80% ethanol and the pH adjusted to 3.5. Dowex 50 (30g, 50–100 mesh) was added to each ethanolic extract and these then packed into glass columns. The columns were washed with 100 ml 80% ethanol at a slow flow rate to remove most of the sugars and other unwanted compounds. The cytokinins were recovered from the Dowex resin using 50 ml 5N NH_4OH. Both the aqueous washes and ammonia fractions for each sample were dried separately under vacuum, and resuspended in 4 ml 80% methanol before being passed through 0.22 μm Millipore filters. After being dried again, each fraction was resuspended in 1 ml 80% methanol.

Each extract was divided into half and these streaked onto a sheet of Whatman No. 1 chromatography paper. The chromatograms were developed using *iso*-propanol:ammonium hydroxide:water (10:1:1). After drying overnight each chromatogram was divided into 10 R_f strips. The R_f strips from one of the chromatograms for each extract was placed in 25 ml Erlenmeyer flasks and 10 ml of medium added [22]. After autoclaving, the cytokinin-like activity for each flask was determined using the soybean callus bioassay [22]. Three approximately equal sized pieces of callus were placed in each flask. The same sized pieces were used for all flasks. After four weeks the pieces of callus in each flask were weighed. Bioassays were repeated twice.

The R_f regions which yielded biological activity in the bioassay were eluted from the second chromatogram using 80% methanol. After filtering (0.22 μm) and drying, the fractions were finally resuspended in 100 μl 80% HPLC-grade methanol and the extract separated using HPLC. A Varian 5000 chromatograph fitted with a 25 cm Supelcosil LC-18-DB (C_{18}, 5μ reverse phase) column and a

gradient elution programme starting at 95% 0.2 M acetic acid, (buffered to pH 3.5 using triethylamine):5% methanol, and moving to a 50:50 ratio over 90 min was used [20]. Ninety 1 ml fractions were collected and then dried in 30 ml Erlenmeyer flasks. The cytokinin-like activity for each fraction was determined using the soybean callus bioassay [22].

3. Results

3.1 Effect of polyamine synthesis inhibitors on cell growth and embryo development

Previous experiments have shown that MGBG, an inhibitor of S-adenosylmethionine decarboxylase, inhibits cell growth and initial embryo differentiation [3]. To further investigate the interrelationship with polyamines, the effect of two inhibitors of polyamine biosynthesis on both cell division in suspension culture and on development of plated embryos, was examined. CHA, an inhibitor of spermidine synthase, inhibited both cell division (Fig. 1A) and embryo development, i.e. formation of polar embryos from globular embryo clumps (Fig. 1B). Although the initial cell number of the various treatments was different, and some fluctuations in cell number are evident (e.g., a decrease at day 14) CHA inhibited cell division during the first 10 days. This was also reflected in the final cell number. Organization of polar embryos proceded at a constant rate throughout the 16 day culture period, and was inhibited by about 40% by 10^{-2} M and 10^{-4}M CHA. DFMO, an enzyme-activated inhibitor of ornithine decarboxylase, did not inhibit cell division (Fig. 1C) and polar embryo formation (Fig. 1D). On the contrary, an increase in embryo development, especially cell division, was noted. Preliminary experiments indicated that DFMA, on the other hand, considerably inhibited both cell division and initial embryo development (data not shown).

A comparative study of the effect of CHA and MGBG (Fig. 2), using standard shake cultures, showed that both inhibitors inhibited the increase in the number of polar embryos and their fresh wt. MGBG was more inhibitory than CHA, resulting in about 50% inhibition at 10^{-3} M. The decrease in embryo number in the control at day 14 is probably due to clumping of single embryos. The data further show a steady increase in growth (fresh wt) in the control during the initial 11 days of culture. This was followed by a second phase of fresh wt increase at day 20. Both phases were inhibited by CHA and MGBG, as mentioned above.

3.2 Polyamine content and metabolism

Non-differentiated embryos, at the globular stage, previously cultured in maintenance medium, were compared with fully differentiated and developed somatic embryos (i.e. plantlets) cultured in regeneration medium. Both the endogenous content of free polyamines (Table 1) and the incorporation of ^{14}C-arginine and ^{14}C-ornithine into polyamines (Fig. 3) were determined. As expected, all 3 polyamines increased on a per unit embryo basis (data not reported), but considerable differences became evident when expressed on a fresh wt basis (Table 1). A high level of free putrescine was found in globular embryos, but plantlets contained only 16% of this level. Spermidine levels in plantlets decreased by about 50%. An increase was however, found in the spermine level. This is reflected in the very marked difference in the ratio of putrescine to spermidine, in the two developmental stages of celery embryos.

The differences in polyamine content over the entire culture period was confirmed by the data from pulse-incorporation of the two major precursors, arginine and ornithine (Fig. 3). About 15% of the label which was taken-up by globular embryos was incorporated into the 3 polyamines. More than 96% of this was detected in the putrescine fraction, compared to ca. 2.5% in spermidine and ca. 1% in spermine. The incorporation of ^{14}C-arginine into putrescine in globular embryos (Fig. 3A) was almost twice that of ^{14}C-ornithine (Fig. 3B), i.e. 110 000 vs 58 000 dpm g^{-1} fresh wt respectively. This is probably due in part to a higher uptake rate of ^{14}C-arginine. However, incorporation of label from arginine was always higher than from ornithine, even when corrected for differences in uptake. The differences in polyamine biosynthesis between globular embryos and plantlets are of special interest when compared with the data for overall polyamine content. Thus, incorporation of label (from both arginine and ornithine) into putrescine was always considerably higher in globular embryos than in plantlets (110 000 vs.

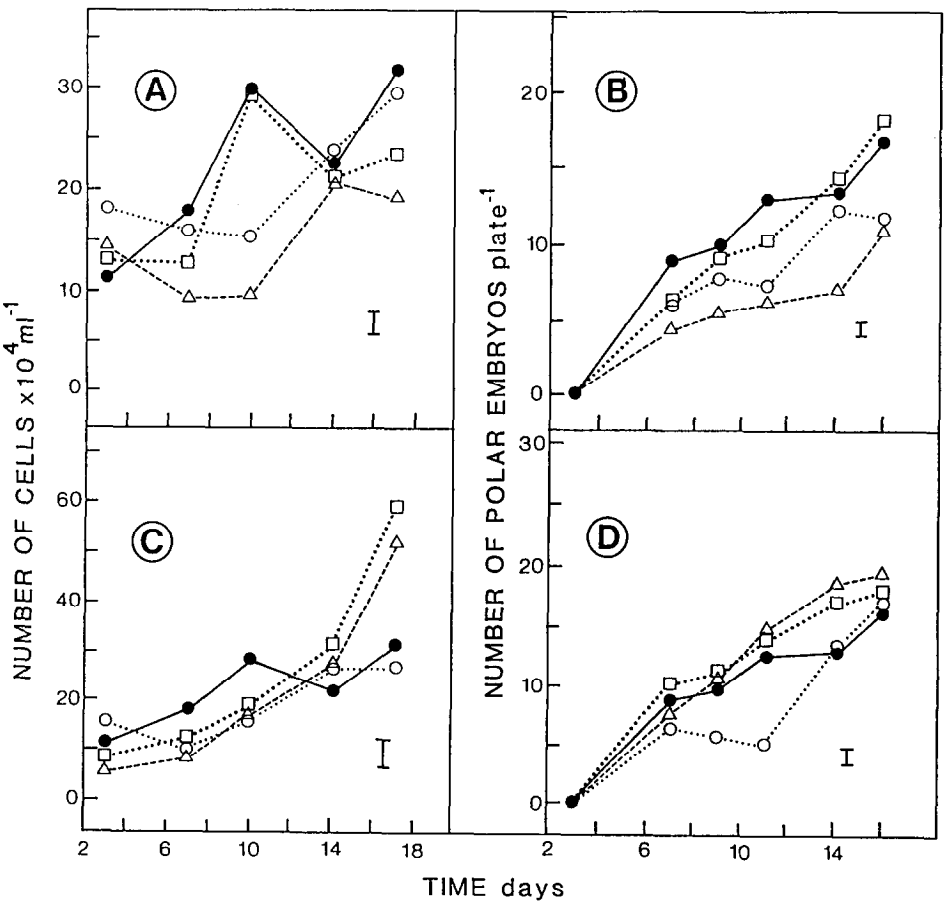

Fig. 1. The effect of CHA (A,B) and DFMO (C,D) on cell division (A,C) and embryo development (B, D) in embryogenic cell cultures of celery. A and C: The < 2000 μm cell fraction was removed from maintenance medium and subcultured in liquid regeneration medium with the inhibitors. The number of single cells plus 2 to 5 cell clumps was recorded. B and D: Individual globular-stage embryos (> 2000 μm cell fraction) were removed from maintenance medium and plated onto semi-solid regeneration medium, with the inhibitors (20 globular embryos per Petri dish). The increase in the number of polar embryos (i.e. heart plus torpedo stages) was recorded. Bars represent the standard error of the mean of all treatments (3 replicates each). ● = control; ○ = 10^{-4}M; □ = 10^{-3}M; △ = 10^{-2}M.

33 000 dpm g^{-1} fresh wt, or 15.5% vs. 3.7% of uptake of arginine, respectively). The reverse was found with incorporation into spermidine and spermine (4 100 vs 4 800 dpm g^{-1} fresh wt, or 3.6% vs. 12.7% of uptake of arginine, respectively). In order to further elucidate the effect of spermidine and spermine biosynthesis inhibitors, the effect of CHA and MGBG on metabolism of ^{14}C-putrescine by embryogenic cultures was studied (Table 2). The results indicate that CHA inhibited spermidine synthesis from putrescine by about 25%. A combination of CHA and MGBG inhibited both spermidine and spermine biosynthesis by about 55% and 50%, respectively.

3.3 Cytokinin content

Size fractions of embryogenic cultures from regeneration medium representing several developmental stages, similar to those used for polyamine analysis, were analysed for cytokinins.

Using paper chromatography, cytokinin-like activity was found at R_f 0.8 and 0.9 of the ammonia fraction of all 3 embryo extracts (Fig. 4). However, cytokinin-like activity in the 500–1400 μm culture fraction, consisting mainly of highly dividing cell clusters and globular embryos, was considerably higher than in the other more differentiated embryo cultures. The 1400–2000 μm and the > 2000 μm size fractions represent mostly torpedo-stage embryos

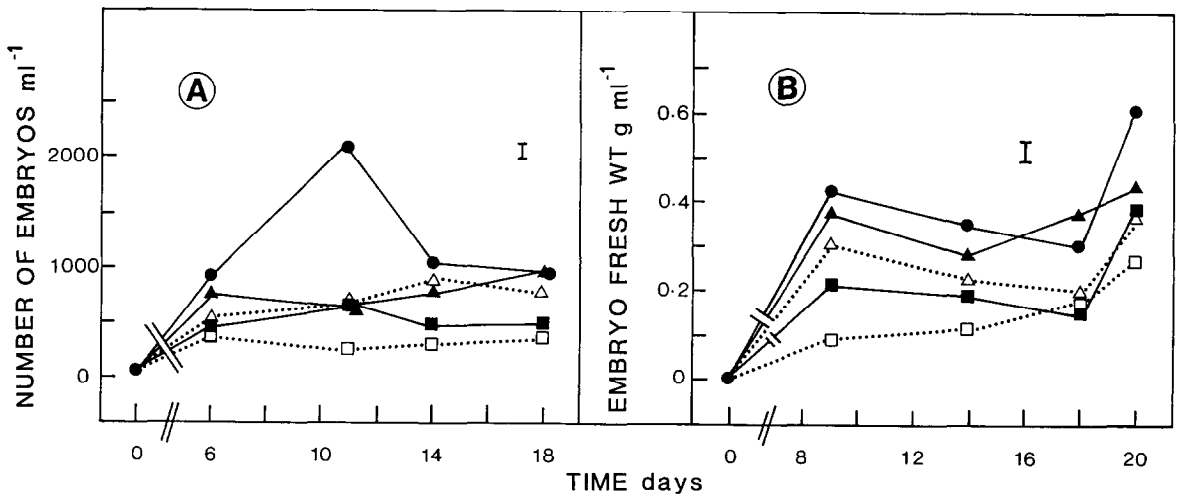

Fig. 2. The effect of CHA and MGBG on (A) embryo formation and (B) embryo fresh wt of embryogenic cell cultures of celery. For (A) the < 2000 μm cell fraction was removed from maintenance medium and sub-cultured in 25 ml liquid regeneration medium with the inhibitors, on a rotary shaker, at an initial 12.5% packed cell volume. Organized embryos (globular and heart stage) were counted regularly in 2 ml suspension samples. For (B) the same procedure was followed except that the fresh wt of embryos was determined. Bars represent the standard error of the mean of all treatments (2 replicates each). ● = control; ▲ = CHA, 10^{-4}M; △ = CHA, 10^{-3}M; ■ = MGBG, 10^{-4}M; □ = MGBG, 10^{-3}M.

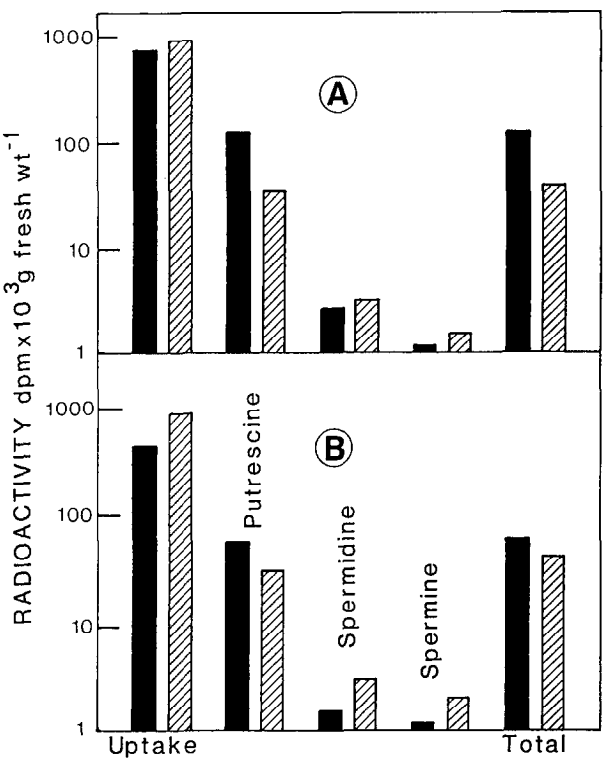

Fig. 3. Polyamine biosynthesis in globular-stage embryos (■) and in fully-differentiated somatic embryos (plantlets) (▨) in embryogenic cell cultures of celery. (A) Globular-stage embryos (cultured in maintenance medium) and fully differentiated plantlets (developed in regeneration medium) were incubated for 5 h with ^{14}C-arginine, followed by perchloric acid extraction, TLC, and radioactivity counting. (B) Same as A, except that cultures were incubated with ^{14}C-ornithine.

Table 1. Free polyamine content of globular embryos and plantlets from embryogenic cell cultures of celery. Globular-stage embryos (cultured in maintenance medium) and fully differentiated plantlets (cultured in regeneration medium) were extracted for free polyamine content

Material analyzed	Polyamine content nmol g^{-1} fresh wt				Ratio
	Putrescine	Spermidine	Spermine	Total	Putrescine/Spermidine
Globular embryos	996 ± 55	181 ± 35	71 ± 10	1248	5.5
Plantlets	159 ± 28	82 ± 15	96 ± 17	336	1.9
Plantlets as % of globular embryos	16.0	45.3	135.2	26.9	

and fully-developed small plantlets, respectively. The biological-active R_f regions were eluted from duplicate chromatograms, concentrated, filtered and then subjected to HPLC fractionation. Bioassay results of all 90 HPLC fractions, again indicate considerable differences in cytokinin-like activity between extracts of the three developmental stages (Fig. 5). In the globular embryos (500–1400 μm cell fraction) most of the activity was coincident with authentic BA. Two additional peaks of activity were detected: one co-eluted with BAR, and the other occurred at a retention time of 79 min and eluted between BA and BAR. While lower, these peaks of activity were also detected in the 1400–2000 μm embryo fraction, but were absent from the fully differentiated embryos (> 2000 μm).

4. Discussion

Removal of celery cell cultures from maintenance medium (containing 2,4-D) and subculture in auxin-free regeneration medium allows for both continuous cell division and polarization of globular embryogenic clumps. This results in formation of heart- and torpedo-stage embryos, and further regeneration of plantlets [25, 26, 27]. The combination of this procedure with the use of defined suspension size fractions allows for determination of changes in the content of endogenous compounds in the various developmental stages of somatic embryos, and of the effect of compounds supplied in the culture medium. In previous studies it was suggested that polyamines may be involved in somatic embryogenesis in celery [3, 4], as was found previously for carrot [6, 11, 13, 21, 23, 29]. This is supported by the present results on the effect of polyamine biosynthesis inhibitors and changes in polyamine content and biosynthesis. CHA and MGBG, competitive inhibitors of spermidine synthase and S-adenosylmethionine decarboxylase respectively, inhibited both cell division in embryogenic cultures of celery and the transition of globular embryos to polar, mature embryos. These two inhibitors markedly inhibited spermidine and spermine biosynthesis. CHA and MGBG also inhibited somatic embryogenesis in carrot and addition of all 3 polyamines restored normal embryogenesis [12, 13]. Addition of spermidine to celery globular embryos promoted embryo maturation and plantlet development [3]. All previous studies on somatic embryogenesis in carrot ascribed the inhibitory effects of various polyamine biosynthesis inhibitors to their effect on putrescine biosynthesis via ADC or ODC, or both. However, these studies differ with regard to the relative role of the two biosynthetic pathways during embryogenic stages. Judged by the effect of DFMA and

Table 2. The effect of CHA and MGBG on spermidine and spermine biosynthesis from ^{14}C-putrescine in celery embryogenic cultures. Globular embryos, cultured in regeneration medium, were incubated for 5 h with ^{14}C-putrescine, without or with the inhibitors, followed by perchloric acid extraction, TLC and radioactivity counting. Data represent dpm in each of the three polyamine spots as percent of total dpm in the perchloric acid extract

Treatment	% of total dpm in extract		
	Putrescine	Spermidine	Spermine
Control	34.5	6.0	1.1
CHA, 10^{-3} M	48.0	4.5	1.0
CHA, 10^{-3} M + MGBG, 10^{-4} M	35.5	2.7	0.5

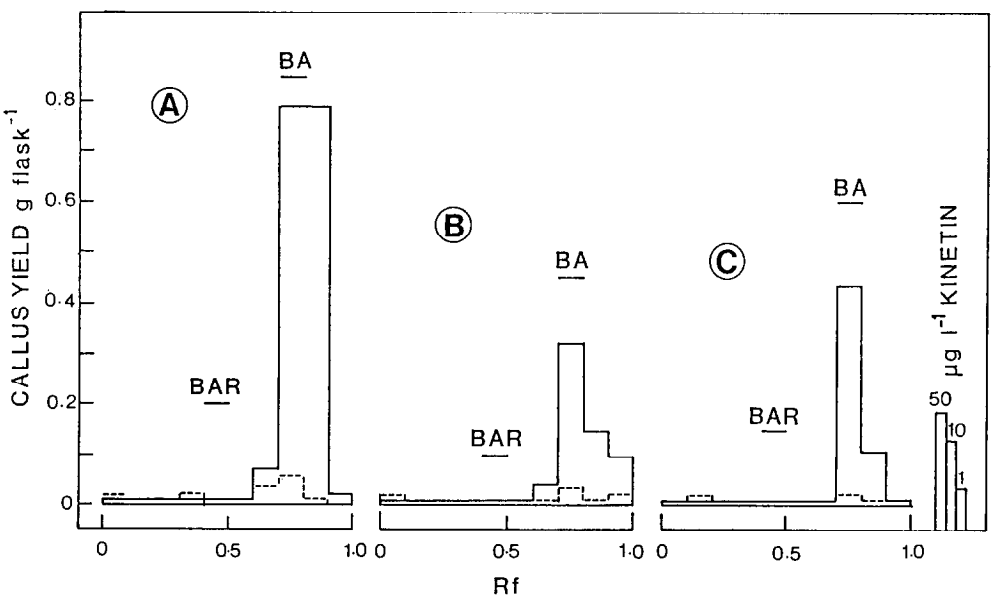

Fig. 4. Cytokinin-like activity in aqueous (– – –) and ammonia (——) extracts of various developmental stages of celery embryos. Extracts were made from the 500–1400 µm culture fraction, consisting mostly of embryogenic cell clusters and globular embryos (A), from the 1400–2000 µ fraction, consisting mostly of torpedo-stage embryos (B), or from the > 2000 µ culture fraction of fully-developed small plantlets (C). Extracts were fractionated on paper and the R_f fractions assayed for biological activity using the soybean callus assay.

enzyme activity measurements, ADC seems to play a major role in carrot embryogenesis, at least during the early stages [11, 13, 29], while ODC activity was detected only in fully mature somatic embryos [29]. A separation of the two phases of somatic embryogenesis in carrot allowed the conclusion that ODC was more active than ADC during the pre-embryogenic phase, while both enzymes displayed similar activities during the embryogenic phase [21]. Activities of ADC and ODC were not measured in the present study, but the similar incorporation rates of arginine and ornithine into polyamines during embryogenesis in celery support the results of Mengoli *et al.* [21]. In agreement with previous studies with carrot [11, 13, 29] DFMO did not inhibit celery somatic embryogenesis which was otherwise inhibited by DFMA (data not shown). It is noteworthy that DFMO slightly stimulated growth and differentiation. This was also found in other studies [10, 21].

The discrepancies in the literature with respect to the relative role of various polyamines and enzymes in somatic embryogenesis may result from different embryogenic phases being analyzed. This was also pointed out by Mengoli *et al.* [21]. In the present study all celery cultures were already committed to somatic embryogenesis (i.e. formation of globular embryos), but both further growth of globular embryos, and their differentiation to polar embryos and plantlets, were dependent on culture conditions.

Using this system, it became evident that polyamine biosynthesis is required for embryo growth and plantlet organization. However, a high putrescine content and biosynthesis relative to spermidine and spermine, are necessary for growth of globular embryos, whereas elevated levels of spermidine and spermine and their biosynthesis were associated with embryo maturation and plantlet formation.

Unlike the role of auxins and specifically 2,4-D, in somatic embryogenesis [5, 17], evidence for a specific role of cytokinins is rather limited. The available evidence indicates that cytokinins are probably required during the induction phase of somatic embryogenesis but not at later phases of embryo development and maturation [8, 9, 17, 28]. Moreover, addition of cytokinins to the medium was found to inhibit somatic embryogenesis in several cases [7, 31]. In the present study the 800 µm embryo fraction which was cultured in regeneration medium with kinetin contained a considerable

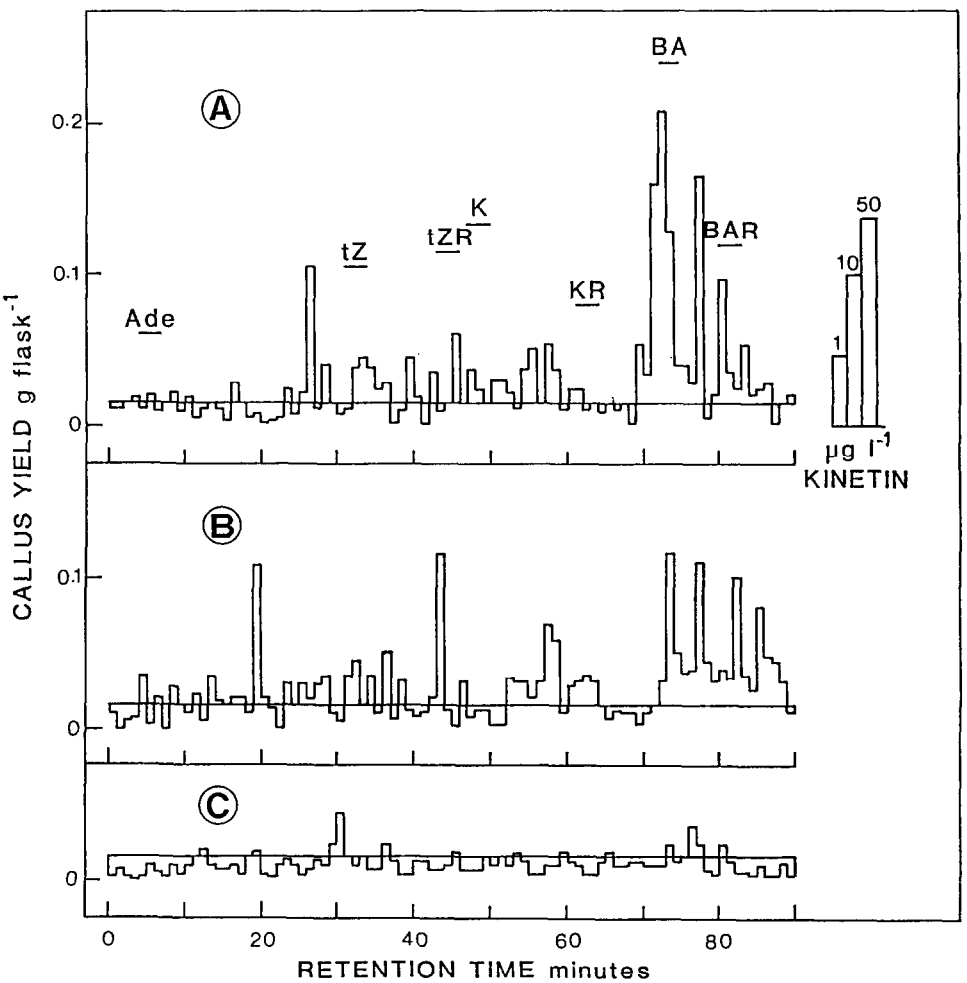

Fig. 5. Cytokinin-like activity in HPLC fractions of the R_f regions 0.8–0.9 of the same embryo extracts as used in Fig. 4.

amount of residual BA, no doubt a carry-over from the BA-containing maintenance medium. BA must have been taken-up, and the biologically-active metabolite appears to be BAR and an unidentified compound with a retention time of 79 min. Even though the regeneration medium contained kinetin, no biological activity coincident with kinetin was detected. Previous studies indicated that kinetin is very rapidly metabolized and could not be detected after 48 h in soybean callus which was routinely maintained with kinetin [15]. BA was metabolized rapidly in the 500–1400 μm fraction of cell clusters and globular embryos. However, no cytokinin-like activity was detected in the $>2000\ \mu$ fraction of the fully-developed embryos. This is in accordance with previous studies indicating high cytokinin levels only in the initial phases of somatic embryogenesis [9]. It appears that there is no clear cut connection between polyamines and cytokinins regarding the process of celery somatic embryogenesis. With respect to the role of cytokinins in the cultures, and its carry over to different developmental phases, it is necessary to establish: why there is a loss in biological activity; whether BA and its metabolites are utilized during embryogenesis, or whether they are metabolized to biologically-inactive compounds? These aspects will be addressed in a subsequent paper.

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