Plant Growth Regulation 3: 319–327 (1985). [117]
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The effects of spermidine synthesis inhibitors on in-vitro plant development

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Key words: Aspen, DCHA, embryogenesis, MGBG, polyamines, spermidine, wild carrot

Abstract. The spermidine synthesis inhibitors methylglyoxal *bis*-(guanylhydrazone) (MGBG) and dicyclohexylammonium sulfate (DCHA) were found to reduce growth and embryogenesis in wild carrot cultures. Cellular polyamine levels were also affected by the inhibitors, with spermidine levels being especially reduced by DCHA. Similarly, MGBG reduced organogenetic development of shoots on excised aspen hypocotyls. These data suggest that the polyamines, especially spermidine, play an important role in the growth and development of plants.

1. Introduction

Elevated levels of the polyamines putrescine, spermidine and spermine and the enzymes leading to their biosynthesis have been associated with growth and differentiation in a wide range of plant, animal, and bacterial systems [7, 8, 18, 21]. Ornithine decarboxylase (ODC), which catalyses the formation of putrescine from ornithine, has an important role in the growth of selected plant species [1, 3, 9] and may be essential for embryo development in animals [6]. Elevated levels of polyamines and arginine decarboxylase (ADC), the enzyme involved in the alternate pathway of putrescine biosynthesis from arginine in plants, have been associated with embryogenesis in cultures of wild carrot [15, 16].

Through the use of specific, irreversible inhibitors of ADC and ODC, ADC and polyamines were shown to be essential for *in vitro* wild carrot embryo formation [4]. Difluoromethylarginine (DFMA) blocked wild carrot embryogenesis by inhibiting ADC activity, which resulted in dramatic reductions of putrescine and spermidine levels in the cells. Embryo development was restored in the DFMA treated cultures by additions of putrescine as well as spermidine or spermine to the culture medium. It was noted, however, that additions of putrescine to the culture medium raised intracellular spermidine more than putrescine levels, suggesting that the return of the spermidine level to near that in the controls might have been responsible for the restoration of embryogenesis. It is possible that ADC activity is essential to development

in wild carrot only because it supplies the substrate, putrescine, for the synthesis of the more important polyamine, spermidine.

Spermidine is synthesized via the enzyme spermidine synthase from putrescine and decarboxylated S-adenosylmethionine (dc-SAM), which contributes an aminopropyl group [18]. Dicyclohexylammonium sulfate (DCHA) inhibits spermidine synthase activity and has been used to lower spermidine levels in bacteria [2, 10]. Spermidine synthesis is also affected by methylglyoxal *bis*-(guanylhydrazone) (MGBG) which inhibits the formation of dc-SAM by SAM-decarboxylase (22). In this study the effects of MGBG and DCHA on wild carrot polyamine levels and somatic embryogenesis were used to provide information on the role of spermidine in growth and development. The effects of these inhibitors on the development of shoots on excised aspen hypocotyls in culture were also examined.

2. Methods

A wild carrot cell line (*Daucus carota* L., obtained from D. Wetherell) maintained as a suspension culture was used as the tissue source. Embryogenesis was induced in the cultures by subculturing the cells into medium lacking exogenous growth regulators as previously described [4]. The time of sub-culture of the cells into the fresh culture medium was defined as day 0 of growth. MGBG, DCHA and spermidine (Sigma) were added to the culture medium at time 0. After 23 days of growth in darkness in 16 × 150 mm roller tubes containing 2 ml medium, the cells were collected on a filter paper and washed. After fresh weight determinations, the embryos in each tube were counted. A structure was scored as an embryo if it was longer than 2 mm and exhibited polarized growth. The data (Figures 1–4) represent the mean ± standard deviation of at least 6 replicates. In order to supply adequate amounts of tissue for analysis, polyamine levels were measured in cultures grown as described above except that 250 ml conical flasks containing 50 ml medium were used. Polyamine levels were measured in cells grown nine days on a gyratory shaker. We previously determined that the polyamine levels are elevated after six to ten days of growth (data not shown).

Polyamine levels were determined in 5% perchloric acid extracts of the cells. Benzoylated derivatives of the polyamines were separated isocratically (55% methanol) on a C18-reverse phase column (Econosphere, Alltech Associates) using a Varian Model 5060 HPLC [4, 5]. It should be noted that DCHA appears to be derivatized along with the polyamines. Unless care is taken in the HPLC separation, the benzoylated DCHA may have a retention time very close to that of the spermidine derivative, resulting in erroneous spermidine levels in DCHA treated cells.

Aspen (*Populus tremuloides* Michx.) seeds were sterilized by stirring in a 0.02% (w/v) of 8-quinolinol sulfate for 5 min. After rinsing with sterile water (3 × 50 ml), the seeds were treated with 0.525% sodium hypochlorite (10%

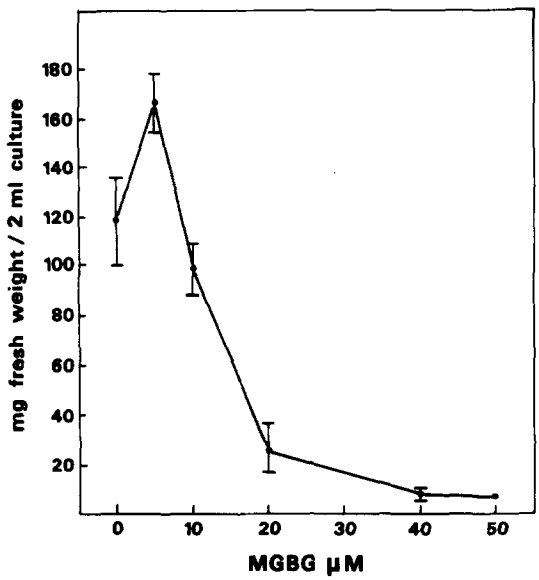

Figure 1. Effect of MGBG on growth of wild carrot suspension cultures.

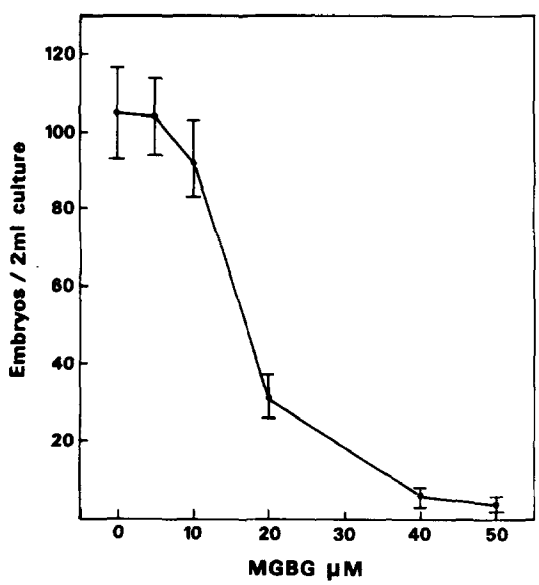

Figure 2. Effect of MGBG on embryogenesis of wild carrot suspension cultures.

Hilex; w/v) for 5 min. In both steps Tween-20 (0.4%) was employed as a wetting agent. Following a rinse with sterile water, seeds were plated on 1% water-agar (Bacto:Difco) and incubated at 5 000 lux (16/8 h photoperiod,

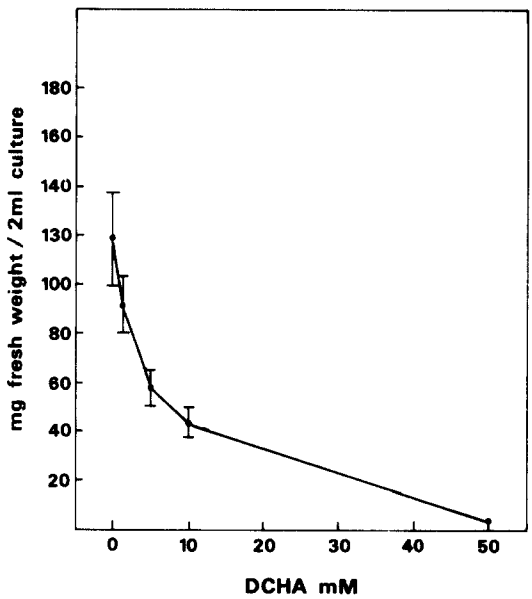

Figure 3. Effect of DCHA on growth of wild carrot cultures.

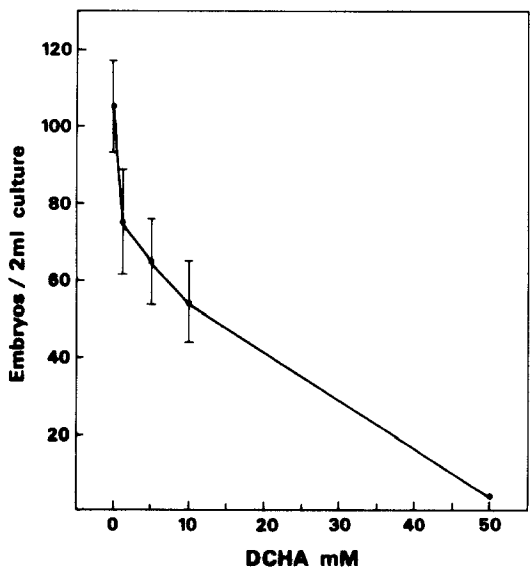

Figure 4. Effect of DCHA on embryogenesis of wild carrot cultures.

cool white fluorescent) and 22 °C. Hypocotyl segments from 4 day old seedlings were excised and placed (8 explants/9 cm Petri dish) on Murashige and Skoog medium [17] (pH 5.8) containing NAA (1-naphthaleneacetic acid,

0.1 mg/l) and BA (6-benzyladenine, 1 mg/l). The medium was solidified with 0.8% agar. MGBG was filter sterilized and added to the autoclaved medium. Following incubation for 4 weeks at 3 000 lux and 23 °C, morphogenesis was scored. The cultures that produced no visible shoot or adventitious buds but proliferated callus were scored as "callus." The explants that differentiated buds and shoots were scored as "shoots," even if some of the buds did not elongate. For each replicated plate in a treatment, this morphogenesis is expressed as a percentage of explants responding.

3. Results

MGBG significantly reduced both embryogenesis and growth when present in the culture medium at concentrations of 20 mM and above (Figures 1, 2). MGBG lowered levels of spermidine and, unexpectedly, putrescine in the cells as compared to controls (Table 1). DCHA also reduced growth and embryo formation in the wild carrot cultures, but higher levels of DCHA were required to result in the same degree of inhibition as obtained with MGBG (Figures 3, 4). DCHA reduced spermidine levels in the cells and also caused a dramatic elevation of putrescine.

Table 1. Effect of MGBG, DCHA and spermidine on wild carrot polyamines. The data represent the mean ± standard deviation of three determinations

Treatment	Putrescine	Spermidine	Spermine
	nmol/gram fresh weight		
Control	266 ± 64	387 ± 57	29 ± 8
MGBG 20 μM	194 ± 34	213 ± 30	40 ± 26
MGBG 20 μM + Spd 1 mM	461 ± 77	2507 ± 693	27 ± 15
MGBG 20 μM + Spd 5 mM	518 ± 99	4300 ± 754	16 ± 10
DCHA 10 mM	837 ± 88	130 ± 7	95 ± 9
DCHA 10 mM + Spd 1 mM	988 ± 160	946 ± 117	17 ± 2
DCHA 10 mM + Spd 5 mM	941 ± 216	1801 ± 185	14 ± 8

Although these inhibitors are presumed to affect growth and embryogenesis by reducing spermidine levels in the cells, and spermidine levels were in fact reduced by the inhibitors in this study, toxic properties of these compounds could be responsible for their effects. Restoration of growth and development in the treated cultures by addition of spermidine to the culture medium would suggest that the inhibitors are affecting only polyamine synthesis and are not toxic to the cells. Spermidine additions to the culture medium at time 0 did result in substantial increases in spermidine in the treated cells (Table 1). In preliminary experiments employing MGBG (20 μM) or DCHA (10 mM), however, spermidine additions have not restored growth or embryogenesis to control levels in the treated cultures (data not shown).

MGBG also reduced callus proliferation, a measure of growth, and shoot induction on aspen hypocotyl explants. Much higher levels of MGBG were necessary to affect the aspen culture than the wild carrot (Figure 5). It was also noted that shoot induction was affected by lower concentrations of MGBG as compared to callus proliferation. Polyamine levels were not determined in the aspen tissues.

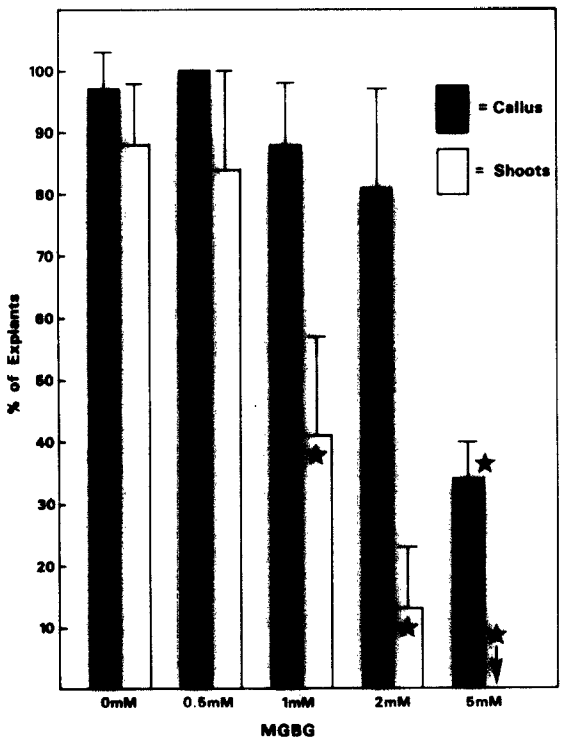

Figure 5. Effect of MGBG on growth and organogenesis of excised aspen hypocotyl explants (expressed as a percent of those explants within a treatment which produced callus or adventitious shoots). The data represent the mean + standard deviation of four petri plates, each containing eight explants. Data were compared by ANOVA and Duncan's Multiple Range Test. '*' indicates a level significantly lower than the respective control, 0 m*M* MGBG ($p < 0.05$).

4. Discussion

MGBG, an inhibitor of SAM-decarboxylase in bacterial and mammalian systems [22], has recently been used in a number of investigations on polyamine metabolism in plants. Hirasawa and Suzuki demonstrated that this compound affected both SAM-decarboxylase activity and the conversion of ^{14}C-putrescine to ^{14}C-spermidine in maize [11]. The reduction of

spermidine levels by MGBG has been demonstrated in germinating barley seeds, and decreased growth and development of cultured chicory explants caused by MGBG has also been reported [20].

In our studies MGBG reduced *in vitro* growth and development in both wild carrot and aspen. Spermidine levels in wild carrot cells were also affected by the inhibitor. Spermidine additions to the culture medium led to elevations of the polyamine in the cells, although in preliminary experiments growth and embryogenesis were not restored by spermidine in MGBG treated cultures.

Spermidine synthase activity and spermidine levels are reduced by DCHA in mammalian cells and bacteria [2, 10]. The results of the present study demonstrate that DCHA decreases spermidine levels in plant cells as well. Also notable was the significant elevation of putrescine in the DCHA treated cells, which apparently accumulated when not being used as a substrate for the biosynthesis of spermidine. This inhibitor also caused dramatic reductions in growth and embryogenesis in wild carrot cultures, suggesting that spermidine plays a key role in these processes. Other investigations have also recently shown that DCHA reduces polyamine levels and embryogenesis in carrot cultures (Z. R. Sung et al., personal communication). Embryogenesis was restored in their DCHA treated cultures by additions of spermidine to the culture medium.

A major role of spermidine in growth and development was suggested by the association of elevated levels of spermidine and SAM-decarboxylase with embryo development in animals [6, 19]. Spermidine concentrations have also been closely correlated with growth in bacteria [2]. The physiological importance of spermidine in plants has been recognized by Kaur-Sawhney et al., in studies on the senescence of oat leaves [12]. The role of spermidine in plant development has also been demonstrated in tobacco cell lines resistant to MGBG, which have elevated levels of spermidine [14]. Abnormal floral development was exhibited in these plants. Earlier studies with wild carrot have shown that spermidine alone can restore embryogenesis in DFMA treated cultures, and that upon the addition of putrescine to these cultures (at levels which restored embryogenesis) spermidine levels in the cells returned closer to control levels than did putrescine [4]. The present study demonstrates a correlation between reduced spermidine levels and inhibition of plant growth and development. It is interesting to note that DCHA, at levels which inhibited growth and embryogenesis, led to decreased spermidine levels but increased putrescine levels in wild carrot. This clearly suggests that spermidine, rather than putrescine, is required for these processes.

The fact that spermidine additions to the culture medium failed to restore embyogenesis in DCHA- or MGBG treated cultures in preliminary experiments suggests that the inhibitors either have effects unrelated to polyamine metabolism or perhaps were simply toxic at the levels employed in these

studies. In addition to the inhibition of SAM-decarboxylase, MGBG has also been reported to affect other enzymes and processes in animal systems [18].

Acknowledgments

The authors wish to thank Shirley Verhagen and Gail Mignon for their valuable help in the tissue culture aspects of this work.

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