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Short Communication

Effect of DL-Alpha-Difluoromethylornithine Pretreatments in Maize Callus Differentiation

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The effect of pretreatments with DL-alpha-difluoromethylornithine (DFMO), an irreversible suicide inhibitor of the ornithine decarboxylase (ODC) activity, in plant differentiation, polyamine (PA) and amino acid contents of maize callus cultures was investigated. This study indicates that DFMO pretreatments can be used to improve regenerative response from maize callus cultures. These findings may also be useful in other recalcitrant cultures.

Key words: Amino acids — Differentiation — Maize callus — Polyamine synthesis — Putrescine.

In vitro culture is an experimental tool in plant breeding and biotechnology only when efficient and reproductible plant regeneration systems are available. Advances in the molecular biology of plants and DNA-manipulation have increased the interest in the differentiation processes themselves and in those achieving plant regeneration.

In recent years the irreversible suicide inhibitor DFMO (Metcalf et al. 1978) has been shown specifically to inhibit plant ODC activities. The inhibition of ODC often causes a decline in growth of plant tissue culture, which can be fully restored on the addition of Put. These aspects have been reviewed in some papers (Altman 1989, Evans and Malmberg 1989, Flores et al. 1989).

In an earlier paper (Tiburcio et al. 1991) we showed that maize callus cultures pretreated with DFMA can increase plant regeneration efficiency. Since DFMO also inhibits Put synthesis via ODC, it would be interesting to study their effects on maize cultures.

Meristematic calluses from W64Ao2 maize inbred line were used as starting plant material for in vitro cultures. This type of callus is an organized tissue obtained from cauline meristem after culture in medium with the auxin 2,4-D. It is derived from the hypertrophic development of a meristem caused by the action of 2,4-D in a specific phase of its development (Santos et al. 1984, Torné et al. 1984, Torné and Santos 1987, 1990). This callus is green and contains an appreciable amount of chlorophyl *a* and *b*. It regenerates plants via organogenesis. Meristematic calluses used in this work were three years old at the time of the experiment and were subcultured every month in glass tubes containing 15 ml of culture medium. The basal medium consisted of a Murashige and Skoog (MS) medium (1962) with some modifications (Torné et al. 1984). For the maintenance of the callus the concentration utilized of 2,4-D was 9 μM. To obtain plant regeneration the concentration of 2,4-D was reduced from 1 μM in the first month to 0 μM in the second month. The pH of the media was always adjusted to 5,8 before autoclaving. All the cultures were incubated at 26–28°C under cool-white fluorescent lights (approx. 90–100 μmol m^{-2} s^{-1}) for 16 h per day (Santos et al. 1984).

Ten calluses for each DFMO treatment, with a minimum of 500 mg fresh weight, were cultured in a medium containing 9 μM 2,4-D (maintenance medium) and 0, 1, 3, 5 or 10 mM DFMO. All the treatments were performed for 3 consecutive months excepting in the case of 10 mM. In this case, the inhibition was performed only for 1 month, because this concentration is considered lethal in long periods of culture. Every 30 days calluses were subcultured in fresh medium. To obtain differentiated structures, at the end of the inhibitory treatments calluses were transferred to a similar medium, but containing 1–0 μM 2,4-D and lacking the inhibitor (differentiation medium).

The total protein content of calluses was determined

Abbreviations: PAs, polyamines; ODC, ornithine decarboxylase; DFMO, DL-alpha-difluoromethylornithine; Put, putrescine; Gaba, gamma-aminobutiric acid; Spd, spermidine; Spm, spermine; Dap, diamino propane; Rr, regeneration rate; Mr, maintenance rate.

according to the method of Bradford (1976) using bovine-gamma-globulin (Sigma) as a standard.

Polyamines were extracted, separated and detected by the method of direct dansylation and thin layer chromatography following procedures detailed in an earlier report (Tiburcio et al. 1985) and quantified using a Perkin Elmer spectrophotofluorimeter.

In order to analyze the endogenous amino acid content, calluses were frozen in liquid nitrogen and stored at −80°C. For each treatment and analysis, 0.5 g fresh weight of callus was used. After freezing, the material was ground in a mortar with liquid nitrogen, adding 4°C bidistilled water in a 1 : 3 ratio (w/v). The homogenate was sonicated and centrifuged at 10,000 rpm for 30 min. Three replicate of each analysis were done.

For free amino acid analysis, one fraction of the supernatant was filtered through an inverted-phase column (Sep-Pak C-18) which was subsequently derivatized with phenylisothiocyanate (PTC) in alkaline medium. The PTC-amino acids thus so obtained were separated by HPLC using a Pico-Tag C-18 column, 300 × 3.1 mm at 46°C. Standard amino acids from the Pierce Chemical Co (Rockford, Illinois, U.S.A.), served for calibration. An auto-amino acid-analyzer HPLC (Millipore Waters, Massachussets, U.S.A.) was used for all the analyses.

Our data show that in vivo treatments with increasing doses of DFMO produced in general a reduction in the growth of the calluses after three months of inhibition. Moreover, the protein content of calluses fell to half that of the control (data not presented). These findings agree with the effect assigned to the inhibitor DFMO.

In our study it may be noted that the total PA content is reduced at the end of the inhibition-experiment in accordance with the inhibitor dosage (see Fig. 1). It was observed that in all cellular fractions Put is the main PA, followed by Spd, Spm and Dap. The acid-soluble hydrolized PAs constitute in general the cellular fraction with the highest PA values regardless of the treatment (data not presented). This finding agrees with that of other authors using DFMO (Galston and Sawhney 1990). In general, when DFMO was increased Put decreases in all the fractions (free and conjugated PAs). However, a relative increase in Put levels for 5 mM DFMO was observed. This increase was smaller than that of the control putrescine level and may be due to the fact that calluses in presence of this inhibitor dosis undergo some negative modifications such as reduction in callus greening or the presence of necrotic zones, after three months of culture. The observed increase in free Gaba levels (three-fold that of the control) can support the latter hypothesis.

The endogenous free amino acid contents of the calluses after the inhibitory treatments are indicated in Table 1. Although the data obtained are insufficient to explain the alterations in the regulatory processes induced by DFMO, they constitute further evidence of the metabolic disorder produced by this inhibitor. The more relevant amino acid concentrations in this calluses are: Ala, Asn, Ser, His, Gln, Glu and Gaba. Nevertheless, the predominant group is formed by Ala and Asn, which represent 80% of the amino acid content and may be related to the C_4 photosynthesis pathway (Hatch 1992). Gln and His tend to enhance their levels according to the inhibitor doses and Asn and Glu

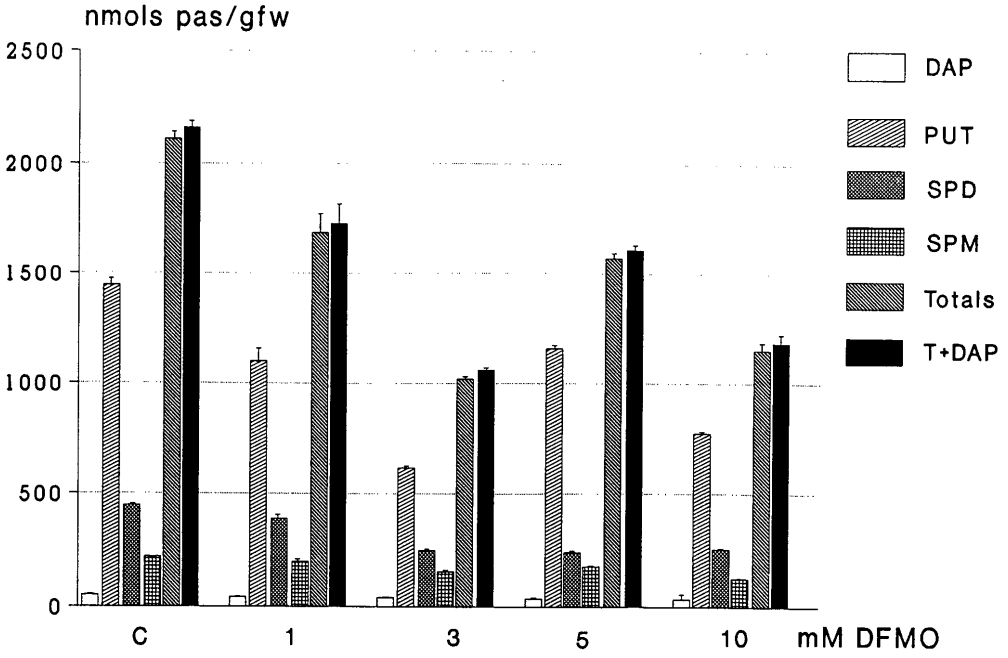

Fig. 1 Endogenous total polyamine content in maize calluses after DFMO-treatments. Bars represent S.E. of the means.

Table 1 Free amino acid content of the DFMO-inhibited calluses

Aa	mM DFMO									
	0		1		3		5		10	
	nmol (g fr wt)$^{-1}$	%	nmol (g fr wt)$^{-1}$	%	nmol (g fr wt)$^{-1}$	%	nmol (g fr wt)$^{-1}$	%	nmol (g fr wt)$^{-1}$	%
Glu	21.54± 2.31	5.14	19.64± 2.56	5.24	11.51± 2.56	3.42	17.08± 5.56	3.58	9.19± 0.73	3.27
Asn	112.91± 4.32	26.91	91.42± 8.5	25.21	62.92± 5.63	18.69	91.86±10.11	19.22	49.44± 1.15	17.47
Ser	19.38± 1.21	4.63	13.15± 2.33	3.63	13.76± 1.23	4.09	12.23± 4.11	2.57	9.04± 0.2	3.21
Gln	4.06± 0.56	0.97	7.29± 1.56	2.02	3.53± 0.56	1.06	19.91± 2.22	4.17	26.84± 4.01	9.34
Gly	3.13± 0.23	0.75	3.76± 1.1	1.04	7.41± 1.13	2.21	10.33± 1.56	2.17	5.18± 0.12	1.84
His	4.11± 1.25	0.98	6.64± 2.56	1.84	22.44± 5.56	6.67	49.24± 8.2	10.31	11.86± 0.61	4.23
Gaba	8.64± 2.36	2.07	12.88± 4.63	3.56	4.89± 1.26	1.46	26.55± 3.23	5.56	7.22± 0.62	2.54
Thr	5.01± 2.22	1.21	3.16± 1.54	0.88	3.81± 0.89	1.14	2.98± 0.56	0.63	1.76± 0.05	0.63
Ala	223.41±15.23	53.25	192.66±10.2	53.11	194.71±13.25	57.83	234.67±19.3	49.11	148.26±11.34	52.02
Val	5.24± 1.36	1.26	3.13± 0.56	0.87	3.71± 0.48	1.11	3.25± 0.23	0.69	2.65± 0.07	0.93

levels tend to decrease. For 5 mM DFMO inhibition, the content of free Gaba was almost three folds higher than that of the control but for 3 mM DFMO it was lower than that of the control. The other amino acids presented non-uniform variations.

The response of the calluses after two months in a differentiation medium without DFMO is given in Table 2. It can be seen that the number of differentiated structures from the 10 mM preinhibited calluses doubled that of the control. In the case of 3 mM it was equivalent to that of the control and in the 5 mM preinhibited calluses it was smaller than in the control.

Nevertheless, the addition of 1 mM of Put in the differentiation medium induced a stimulatory effect in the regeneration process of the pre-inhibited calluses, increasing the number of buds and consequently the regeneration rate (Rr). The optimal response (see Table 2) was obtained by pretreatment with 10 mM DFMO and subsequent addition of 1 mM Put (Rr=10.9; sixfold the control). The pretreatment with 3 mM DFMO and the addition of 1 mM Put was also effective (Rr=7.1; fourfold the control). The response showed a sharp decrease when Put was increased at 3 mM (Rr=0.8). However, when 1 mM Put was added to non preinhibited calluses, the regeneration was not improved.

The maintenance rate (Mr) gave us information about the preservation of regenerating calluses after two months in differentiation medium. It can be seen (Table 2) that the pretreatment with 3 mM DFMO produced the best response (Mr=0.5) followed by 1 mM, which is equivalent to the control (Mr=0.3). Putrescine enhances this rate and prevents callus degeneration in the case of 3 mM (Mr=0.7) and 10 mM DFMO (Mr=0.6). This rate is lower than the control in the other cases. In general, it may be said that the effect of DFMO preinhibition with the subsequent addi-

Table 2 Response of the calluses after two months in differentiation medium without DFMO and effect of Put addition

DFMO	PUT	C	B	P	GC	T	Rr	Mr
10	—	5	12	4	0	16	3.2	0
10	1	5	51	3	3	54	10.9	0.60
10	3	5	4	0	0	4	0.8	0
5	—	6	2	0	0	2	0.33	0
5	1	7	5	2	0	7	1	0
3	—	6	4	4	3	8	1.33	0.5
3	1	7	48	2	5	50	7.1	0.7
1	—	6	3	2	2	5	0.8	0.3
1	1	6	16	13	1	29	4.8	0.16
0	—	6	4	6	2	10	1.7	0.3
0	1	6	6	4	0	10	1.7	0

DFMO and Put concentrations are expressed in mmol liter C=number of calluses on differentation medium; B=buds; P=plantlets; GC=green calluses; T=B+P; Rr (regeneration rate)=T/C; Mr (maintenance rate)=GC/C.

tion of Put is related to an increase in the number of buds (which will subsequently produce plants) at the end of the regeneration period. This multiplicative effect on the production of buds can be compared to the effect produced by the hormone-combination of NAA and 2iP when these are added to the differentiation medium as has been demonstrated in previous experiments (Torné and Santos 1987).

Comparing the best results of differentiation obtained, it can be stated that higher doses (10 mM) and a shorter period of inhibition produced a regenerative rate (Rr) which doubled that of the control. By contrast, 3 mM inhibition for 3 months produced a Rr that was slightly lower than the control. In both treated calluses a similar PA level can be observed. The results of differentiation obtained suggest that a DFMO-inhibitory pretreatment can enhance the differentiation process in maize cultures especially if the dose of inhibitor is suitable.

Although it is as yet no possible to demonstrate a causal relationship between the inhibitor effects and the specific morphogenetic process, this study indicates that DFMO pretreatments may be used to increase regenerative response from maize callus cultures. Finally, these findings provide information that may be useful in other plant biotechnological systems or recalcitrant cultures.

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