Euphytica 35 (1986) 161–168

ETHYL-METHANE-SULPHONATE EFFECTS ON ANTHER CULTURES OF *NICOTIANA TABACUM*

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Received 28 December 1984

INDEX WORDS

Nicotiana tabacum, tobacco, anther culture, ethyl methane-sulphonate, mutagenesis.

SUMMARY

Anthers from *Nicotiana tabacum* L., cv. Wisconsin-38, were treated with various concentrations of Ethyl-methane-sulphonate (EMS) during different application times.

Low doses and short application times showed increases in the number of anther producing plantlets, average number of plantlets per anther, and early plantlet production. The mutagen treatment increased the variance of protein electrophoresis fractions and chlorophyll content of the plantlets.

INTRODUCTION

Since the initial report by GUHA & MAHESWARI (1964) appeared, anther culture and haploid production have been successful in a large number of plant species and genera (MAHESWARI et al.,1982).

Anther and pollen culture could be of significant value to obtain haploid plants for breeding, particularly in species like *Nicotiana tabacum* where such cultures are relatively easy (COLLINS & LEGG, 1977).

Mutagens have been used in breeding to change one or more characteristics and to increase genetic variability (KONZAK, 1984). Haploid plants simplify the detection of mutants because recessive mutations are not masked by dominant alleles, (MURASHIGE, 1978). Thus, haploids have been used to study spontaneous and induced mutagenesis (CONSTANTIN, 1981; PRZEWORNY et al., 1980). The effects of mutagens on androgenesis have been reported in treatments with irradiation (MONDEIL, 1974) and chemical mutagens (VAGERA, 1978; VAGERA & NOVAK, 1979).

The use of haploids in mutagenesis and selection experiments offers the advantages that the researcher can introduce genetic variability with an appropriate mutegen and screen directly for the desirable mutant phenotype. In this paper we studied the effects of EMS treatments on anther culture as well as the variation in chlorophyll content and proteins.

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MATERIAL AND METHODS

Material. Anthers were obtained from flower buds of *Nicotiana tabacum* cv. Wisconsin-38, grown under glasshouse conditions (27 °C day, 24 °C night, 16 h light), in pots with sand: peat, 1:1, watered every 7 days with Hoaglands No 2 solution.

Methods. Anther culture: Flower buds collected when the petal length was equal to the sepal length (PELLETIER & ILAMI, 1972), were surface sterilized by inmersion in sodium hypochlorite solution (7% v/v) and subsequently rinsed with sterile water. Under sterile conditions anthers were excised and soaked in a filter sterilized mutagen solution (25 μ mesh filter). The anthers were then rinsed three times in sterile water and inoculated in petri dishes (10 cm. diameter) with 0.8% agar solidified medium containing the salts and vitamins of MURASHIGE & SKOOG (1962), sucrose 2% and pH adjusted to 5.6.

The treatments were carried out using 30 petri dishes with 10 anthers in each, which were kept at 27 °C, with 16 h. light at 2000 lux (Grolux tubes). The number of anthers producing plantlets were counted 40, 50, 60 and 90 days after the start of anther culture. The number of plants per productive anther was evaluated after the 90 days countig.

Electrophoresis. 10 grams of fresh leaf tissue were homogenized with polytron in 10 ml of 0.26 M, triphosphoric buffer pH 6.9. The homogenate was filtered through a 150 μ mesh filter and centrifuged at 10000 *g* for 20 min. The supernatant was dialyzed with the same buffer at 1:10 dilution, and was run in a polyacrylamide gel electrophoresis according to *Conejero & Semanscik* (1977) at 4 °C, 18 mA, 200–240 min. Twenty electrophoretic determinations were made in haploid non-treated and twenty in EMS treated ones. A quantitative study was made in the eight bigger fractions quantified by Densitometer (Beckman R-115).

Chlorophyll content. Leaves from haploid plants, growing under the glasshouse conditions mentioned above, were collected when their first flowrs opened. Chorophyll content was determined in twenty plants of each group; non-treated (control), treated with 0.1%, and with 0.01% of EMS for 1 hour. Each of the twenty samples consisted of three leaf disks of 11 cm diameter. Chlorophyll was extracted with 85% acetone, keeping the disks in the dark at 4 °C for 48 h. All manipulations were carried out under low illumination at 4 °C. Chlorophyll content was determined by the ARNON'S method (1949).

RESULTS AND DISCUSSION

EMS effect on anther culture. Table 1 shows EMS treatment effects on the percentage of anthers that produce plantlets and on the number of plantlets per productive anther. The increase in concentration and application times results in a progresive decrease in the number of productive anthers and in the number of plantlets per productive anther.

In our conditions EMS 1% v/v seems lethal to the anthers in 1, 2 and 3 hours of

Table 1. EMS effect on plantlet yield per anther.

Time of treatment	EMS concentration % (v/v)	% Anthers producing plantlets	Average number of plants per productive anther
Control	–	7.33	6.1 ± 0.6
1 hour	0.005	7.0	14.3 ± 1.1*
	0.01	11.66*	8.7 ± 0.9
	0.05	7.0	5.5 ± 1.4
	0.1	6.66	4.0 ± 1.4*
	0.5	4.33*	2.2 ± 2.2*
	1	0.6*	1.0 ± 0*
2 hours	0.005	13.33*	12.4 ± 0.9*
	0.01	7.0	5.9 ± 1.3
	0.05	5.6	3.0 ± 2.5*
	0.1	4.33*	2.5 ± 2.6*
	0.5	0.6*	1.0 ± 0*
	1	0.3*	1.0 ± 0*
3 hours	0.005	12.0*	6.5 ± 1.2
	0.01	4.0*	4.2 ± 2.8*
	0.05	3.3*	1.1 ± 0*
	0.1	3.3*	1.2 ± 0*
	0.5	0.6*	1.0 ± 0*
	1	0.0*	0.0 ± 0*

* Values significantly different from control, according to t test.

treatment. The results show that low EMS concentrations and short application periods have a significant positive effect on the number of anthers producing plantlets (EMS at 0.1%, 1 h; and 0.05% 1 and 2 hours) and on the number of plantlets obtained per productive anther (EMS at 0.005% 1 and 2 hours). This stimulating effect also produces early induction of androgenesis. The percentage of productive anthers after

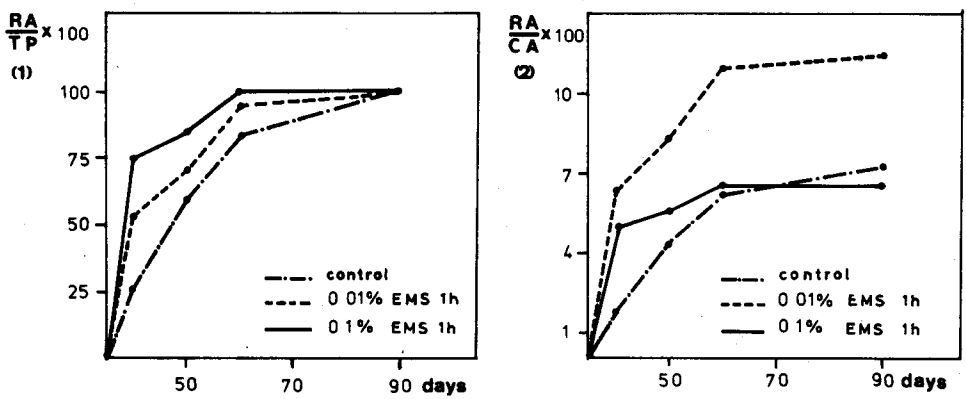

Fig. 1. EMS effect on early androgenesis. Left: % of responding anthers (RA) is proportion to the number of total productive anthers (TP) at the end of experiment. Right: % of responding anthers (RA) in proportion to the total cultured anthers (CA).

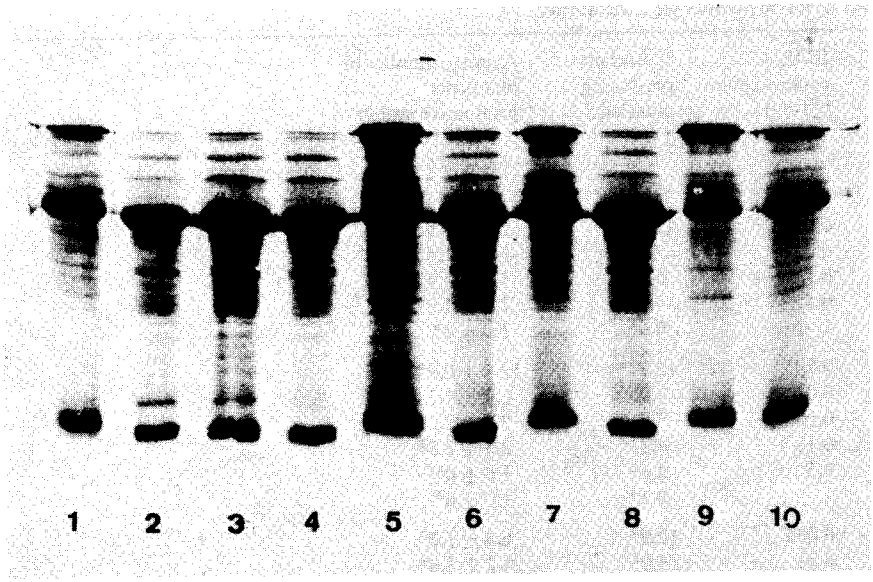

Fig. 2. Electrophoretic profiles of leaf proteins of 10 haploids plants. 1–4, samples of haploid plants from non-treated anthers: 5–10, samples from EMS treated anthers (0.1% v/v 1 hour).

30, 50, 60 and 90 days of culture is higher in anthers treated at low EMS concentrations and short application periods (Fig. 1).

The stimulating effects of low doses of mutagens has been reported by BAJAJ et al. (1970). and MODEIL (1974), with ionizing radiation treatments on seeds, plantlets, and callus, and by VAGERA (1972) and VAGERA et al. (1976) with chemical mutagens in experiments where seeds of *Nicotiana tabacum* were treated, and anthers from plants that emerged from these seeds, were cultured 'in vitro'. In these experiments, lower mutagenic treatments, produced a percentage of embryogenic anthers superior to the control and an increase in the average number of true leaves in haploids derived from the treated plants, corresponding to a smaller number of plantlets per embryogenic anther. The increase in genetic heterogeneity of pollen grains due to the application of mutagen was claimed as a possible explanation for this effect.

Direct application of mutagen to anthers has been reported as a disadvantageous method by RAINA & IYER (1982) who observed a considerable decrease in anther response in 2 and 6 hour treatments at 0.025% EMS.

The results reported here, show that mutagen treatments of low concentrations for short periods of time have a significantly favourable effect on androgenesis in directly treated anthers.

According to SUNDERLAND (1970) and SUNDERLAND & WICKS (1971), only a few anthers produce plantlets in *Nicotiana tabacum*, compared to the percentage of anthers with early stages of embryo development, and only about 0.005% of pollen grains ultimately give rise to haploid plants. The competition among embryoids in the anther has been suggested as a possible explanation.

The stimulation of androgenesis due to low doses of chemical mutagen could be

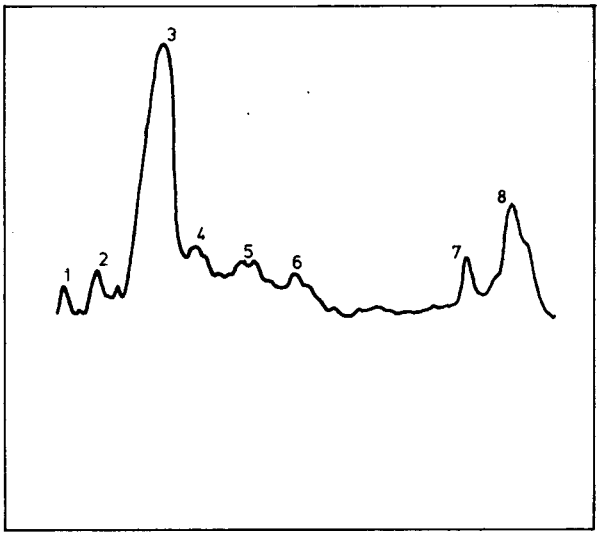

Fig. 3. Densitogram profils of the electrophoregram of leaf proteins of a control haploi. 1, 2, ..., 8: Protein fractions.

explained by a reduction of the number of induced embryoids because of the effect of treatment that causes a decrease in the competition at the first stages of embryo growth, allowing the complete development of a greater number of embryoids. The reduction of competition would also enhance the early emergence of plantlets. Higher concentration of mutagen strongly decrease the viability of embryoids and therefore plantlet induction is drastically reduced.

EMS effect on electrophoretic patters of proteins. Fig. 2. shows the electrophoretic profiles of proteins from several haploid plants obtained from anthers treated with EMS. Fig. 3 shows a densitogram profile of a control plant. Some differences may be observed between plants derived from EMS treated and from non-treated anthers. The main variations in electrophoretic profiles are quantitative. However, appearance

Table 2. EMS effect on leaf electrophoretic pattern of proteins in gel electrophoresis.

		% Densitogram surface							
		PF1	PF2	PF3	PF4	PF5	PF6	PF7	PF8
Haploids from	$\bar{x}$	3.08	3.32	29.37	6.17	3.5	6.58	5.00	17.74
non treated anthers	s^2_{nt}	0.44a	1.00a	1.11a	0.80a	0.96a	1.22a	0.66a	2.51a
Haploids from	$\bar{x}$	3.35	3.35	30.36	6.62	3.76	6.87	5.46	18.50
EMS treated anthers	s^2_t	0.70a	1.45a	4.79b	1.43a	1.15a	1.74a	2.14b	2.32a
(0.1% 1 hour)	s^2_t/s^2_{nt}	1.59	1.45	4.32	1.79	1.20	1.43	3.24	0.92

s^2_t: Variance of EMS treated samples; s^2_{nt}: Variance of EMS non treated samples.
Data in the same column without a common subindex significant differ at 95% of probability, variances have been compared according the F test.

Table 3. Number of haploid plants and chlorophyll mutants.

Time of treatment	EMS concentration % (v/v)	Number of haploid plants obtained	Number of chlorophyll mutants	
			albinos	others
Control	–	122	1	1
1 hour	0.005	300	2	2
	0.01	278	2	5
	0.05	115	3	7
	0.1	72	2	6
	0.5	26	0	2
	1	2	0	0
2 hours	0.005	397	2	6
	0.01	112	3	7
	0.05	42	1	4
	0.1	30	0	2
	0.5	2	0	0
	1	1	0	0
3 hours	0.005	214	3	10
	0.01	42	2	4
	0.05	11	0	2
	0.1	9	1	0
	0.5	2	0	0
	1	0	0	0

and disappearance of some low density bands may be observed, even though the visual observation of electrophoretic gels and the desitogram resolution do not allow to clearly distinguish whether these alterations are qualitative of quantitative.

Table 2 shows the average values of the relative proportion of densitogram surfaces of the eight electrophoretic components in treated and non-treated haploids. The mean values are similar, but the variance is greater in all fractions of plants derived from EMS treated anthers (except P.F. 8). In P.F. 3 and P.F. 7, the variance values of the above mentioned plants are significantly higher than those derived from non-treated anthers.

This increase in variance values of protein fraction might reflect a higher variation in the treated population.

EMS effect on chlorophyll content. Changes in leaf colour have often been related to mutagen treatments. Some variegated plants and others with low chlorophyll content were obtained. Table 3 shows the effect of mutagen treatments on the number of albinos and other chlorophyll mutants obtained from anthers. There was a clear relationship between concentration and application times of mutagen and mutation frequency.

The chlorophyll content values are reported in Table 4. Mean values show no significant differences, but variances are clearly higher in plants derived from treated anthers and significantly different between plants derived from 0.1 EMS treated anthers and those derived from non-treated ones.

Table 4. Mutagen effect on chlorophyll content.

Mutagen concentration EMS (v/v)	Chl. a µg/cm²		Chl. b µg/cm²		Total Chl. µg/cm²	
	$\bar{x}$	s^2	$\bar{x}$	s^2	$\bar{x}$	s^2
Control	13.11a	7.8a	4.83	2.11a	17.94a	13.07a
0.01	15.63a	10.80ab	4.96a	3.10ab	19.59a	16.81ab
0.1	12.40a	20.17b	5.08a	4.80b	17.48a	27.08b

Data in the same column without a common subindex significantly differ at 95% of probability, variances have been compared according the F test.

Higher values of variance in haploids from treated anthers show a significant mutagen effect on variation. These results and the increase in embryogenic anthers, at low doses of mutagen treatment, show that direct treatments of anthers with chemical mutagens, may be an effective stimulus to androgenesis and the enhanced genetic variability of haploids.

ACKNOWLEDGEMENTS

We would like to thank Mrs Rosario Millan for her invaluable technical assistance in all this work and the assessment of Mrs N. Durán for editorial assistance.

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