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Embryo Culture as a Method of Securing Viable Mutants in *Vigna sinensis* var. Black and *V. radiata* (B-105) after EMS Treatment

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The present investigation deals with a comparative analysis of the effect of EMS on embryos grown *in vitro* prior to transplantation and on the seeds sown directly in the field. Treated embryos were first grown *in vitro* in White's medium and later in field conditions. This method is more effective in securing a high percentage of germination, irrespective of the dose applied, as compared to that of seed treatment. Chlorophyll and morphological mutants were recorded in M_2 and M_3 generations. In *Vigna sinensis* var. Black, a remarkable long pod mutant from EMS treated embryos followed by *in vitro* culture, was obtained. In *V. sinensis* and *V. radiata*, high percentages of tall, dwarf, early flowering mutants were isolated. The present data indicate that mutagen treatment of embryos followed by growth of the seedlings in culture medium prior to transfer to the field yields a wide spectrum of mutants.

Key Words: Embryo Culture, *In vitro* mutagenesis, Embryo mutant, *Vigna sinensis*, *Vigna radiata*

Introduction

Considerable work on induced mutation in crop species has been carried out by various workers (Ashri & Levy 1974, Ehrenberg 1971, Gustafsson 1954, Nilan 1972, Sigurbjornsson & Micke 1969, Swaminathan 1969). Of the different chemical mutagens used, ethylmethane sulphonate has been found to be extremely potent. It has been utilized to influence germinability, vegetative and floral characters, quality and yield of grains and other features of economic importance. Several legumes have been exposed to mutagenic agents for isolating mutants of commercial value (Appa Rao & Reddy 1975,

1976, Blixt & Gottschalk 1975, Gottschalk 1969, Appa Rao & Jana 1976, Jambhale et al. 1977, Sharma 1969). One of the serious limitations of the study of induced mutations is the low survival rate of mutants. Even if the viability of the seeds is not effected, seedling lethality is a common feature.

Embryo culture technique offers immense possibilities of overcoming some of the limitations of non-viability and seedling-lethality. The nutritional deficiency responsible for either non-viability and seedling-lethality can be met by supplying nutrients *in vitro*. Comparatively healthy seedlings can be obtained and transferred to the field. A large number of mutants can thus be obtained from which subsequent selection can be effectively made.

The present work was carried out to explore the possibility of utilizing the embryo culture technique for securing mutants in *Vigna sinensis* var. Black and *V. radiata* (B-105). For a comparative assessment of the efficacy of this method against direct sowing in the field, mutagen-treated excised embryos were grown *in vitro* prior to transplantation.

Materials and Methods

One hundred seeds of uniform size each of *V. sinensis* and *V. radiata* were soaked in distilled water for 6 hours and then treated for 12 and 24 hours with freshly prepared solutions of 0.25%, 0.5% and 1% EMS (ethylmethane sulphonate) at 20°C with intermittent shaking. The seeds were washed in running tap water for one hour and then sown in the field. All the M_1 plants were selfed, and harvested individually at maturity. In the following season, M_2 rows of 50 plants were sown. Chlorophyll and macromutations were scored. M_3 progenies were obtained from selected M_2 seeds in a similar way. A randomized block design with 4 replications was adopted in the M_2 generation. The first dates of anthesis of randomly selected plants in each replication were recorded. Other morphological characters were also recorded from each replication.

For embryo culture one hundred selected and sun dried seeds of uniform size of *V. sinensis* and *V. radiata* were soaked in distilled water and surface sterilized with mercuric chloride (0.1%). After washing the seeds several times in sterile distilled water, the embryos were carefully excised under aseptic conditions. The embryos were then soaked in freshly prepared EMS solution (sterilized through millipore filter) of different concentrations at 20°C for 12 and 24 hours. The embryos were then washed thoroughly with sterile distilled water and transferred aseptically to White's nutrient medium (1963). The cultures were maintained at 25±1°C under 10 hr light (2000 lux) and 14 hr daily dark periods. The seedlings developed in culture were transferred carefully to the field after two weeks. An evaluation of the mutants was made under field conditions.

In both sets of experiments, observations were recorded for different characters at M_1, M_2 and M_3 generations. The M_2 seeds were harvested from each M_1 plant on individual plant basis. The M_3 seeds obtained from each M_2 plant were harvested separately and data on the quantitative characters were recorded from 20 plants selected randomly from each plot. To analyze the rate of induced variations of quantitative characters in both the sets of experiment, standard errors were calculated and frequencies of mutant characters were expressed in percentage.

Results and Discussion

(i) *Viability and seedling survival*

With increase in concentration of EMS there was a gradual fall of seedling growth

under both field and *in vitro* conditions. At higher concentrations i. e., with 0.5% EMS treatment, embryos of *V. radiata* showed high percentage of germination *in vitro* as compared to direct sowing (table 2). Otherwise the response of the two species was more or less identical. Most of the seedlings from the low concentration EMS treatments survived. However at 1% EMS, the rate of survival was very low in *V. radiata* and *V. sinensis* (2% in *V. radiata* and 8% in *V. sinensis* respectively, *see* table 1). Following *in vitro* growth, the survival rate of the seedlings reached 24% and 30% in these species respectively (tables 1 and 2).

It may be inferred that EMS did not inhibit germination and survival of seedlings at low concentrations. At higher concentration its toxic effect was manifested not during germination but in seedling growth. This effect was compensated by the nutrients in the culture medium.

A comparative analysis of the effect of EMS on the two materials showed that EMS in the case of *in vitro* culture affects the survival rate of mutated seeds in general. The low lethality following *in vitro* treatment could be attributed to different factors like optimum hydration, temperature and light, easily assimilable carbohydrate in the form of sucrose, absence of injurious effects of treated endosperms and removal of seed coat, accelerating growth of the embryonal axis. A similar observation was made in rice (Bhaduri & Brahmachari 1975) where these factors have been considered responsible for accelerated survival.

Table 1 *Effect of EMS on seedling survival and lethality in* Vigna sinensis

Treatment (concentration and time)	In field condition		In culture condition	
	Germination (%)	Survival (%)	Germination (%)	Survival (%)
Control	94	92	92	90
0.25%:24 hr	74	64	80	74
0.25%:12 hr	86	68	98	86
0.5 %:24 hr	64	54	68	63
0.5 %:12 hr	50	42	56	50
1 %:12 hr	24	8	34	30

Table 2 *Effect of EMS on seedling survival and lethality in* Vigna radiata

Treatment (concentration and time)	In field condition		In culture condition	
	Germination (%)	Survival (%)	Germination (%)	Survival (%)
Control	92	88	94	91
0.25%:24 hr	68	64	76	74
0.5 %:24 hr	24	24	42	40
1 %:12 hr	6	2	28	24

Table 3 *Different EMS mutants of* Vigna radiata *following* in vitro *growth or direct sowing (in percentage)*

Generation	Treatment		Light green	Xantha	Tall	Dwarf
	0.5% EMS	direct	4	3	6	9
	24 hr	*in vitro*	—	—	4	5
M_1	0.5% EMS	direct	2	—	4	1
	12 hr	*in vitro*	—	—	5	8
	1% EMS	direct	—	2	—	5
	12 hr	*in vitro*	—	—	—	7
	0.5% EMS	direct	—	—	9	10
	12 hr	*in vitro*	—	—	12	—
M_2	0.5% EMS	direct	—	—	3	6
	12 hr	*in vitro*	—	—	4	7
	1% EMS	direct	—	—	—	5
	12 hr	*in vitro*	—	—	—	14
	0.5% EMS	direct	5	—	14	11
	24 hr	*in vitro*	—	—	20	—
M_3	0.5% EMS	direct	2	—	—	3
	12 hr	*in vitro*	—	—	—	10
	1% EMS	direct	—	—	—	6
	12 hr	*in vitro*	—	—	—	12

Table 4 *Different EMS mutants of* Vigna sinensis *following* in vitro *growth or direct sowing (in percentage)*

Generation	Treatment		Light green	Xantha	Tall	Dwarf
	0.25% EMS	direct	1	—	—	—
	12 hr	*in vitro*	—	—	—	—
M_1	0.5% EMS	direct	8	2	9	5
	24 hr	*in vitro*	3	—	12	—
	0.5% EMS	direct	6	3	—	1
		in vitro	—	—	—	16
	0.25% EMS	direct	8	—	—	—
	12 hr	*in vitro*	—	—	—	—
M_2	0.5% EMS	direct	7	5	2	1
	24 hr	*in vitro*	2	3	11	—
	0.5% EMS	direct	5	2	—	3
	12 hr	*in vitro*	—	—	—	12
	0.25% EMS	direct	13	—	—	—
	12 hr	*in vitro*	—	—	—	—
M_3	0.5% EMS	direct	14	2	16	4
	24 hr	*in vitro*	1	—	2	1
	0.5% EMS	direct	3	18	16	3
	12 hr	*in vitro*	—	—	—	11

(ii) Other mutants

The number and kinds of mutants obtained in *V. radiata* and *V. sinensis* following EMS treatment are recorded in tables 3 & 4. Tall, dwarf, small leaved, yellow and albino mutants were obtained in plants raised from embryo cultures as well as from directly sown seeds. For example, with 0.5% EMS given for 24 hours, tall mutants were obtained in large number in M_2, and M_3 whereas with directly sown seeds almost similar frequency was recorded only in the M_3 generation. Ten and twelve dwarf mutants were obtained in M_2 and M_3 from *in vitro* treatments. From a comparative analysis, the effect in M_1 was not regarded as very relevant as in that generation the manifestation was purely of generalized nature, several genes being affected simultaneously.

(iii) Morphological mutants

In *V. radiata* treatment with lower and higher EMS concentrations revealed a significant difference from the control in the average number of grains per pod, pod size and secondary branches (tables 5–6). In plants raised from embryo culture, pod size and number of grains per pod were greater than in those raised from seeds directly sown in the field.

In *V. sinensis* the plant height showed a significant increase both in direct sowing and *in vitro* treatment in M_2 generation at 0.5% EMS, given for 12 hours (table 6). The selfing of such individual plants in the M_3 generation showed the same effect on height which was more pronounced in *in vitro* raised seedlings transplanted to soil. Similar response was noted in treatment with 0.25%

Table 5 *Morphological characters obtained from direct sowing and after* in vitro *growth in M_2 and M_3 plants of* Vigna radiata

Generation	Treatment	Height	Nodes	Secondary branches	Leaflets	Pod size	No. of grains	Days to flower
M_2	Control (in direct sowing)	48.0±0.24	8±0.24	—	10±0.31	6.1±0.19	8±0.12	60
	(*in vitro*)	47.3±0.28	8±0.50	—	10±0.42	5.9±0.17	8±0.44	61
	0.25%EMS (in direct sowing)	26.0±0.34	7±0.07	—	8±0.21	7.0±0.21	9±0.20	53
	24 hr (*in vitro*)	40.0±0.74	8±0.24	4±0.32	11±0.42	6.0±0.17	9±0.20	51
	0.5%EMS (in direct sowing)	31.0±0.26	8±0.34	—	11±0.08	7.0±0.19	10±0.19	45
	24 hr (*in vitro*)	36.8±0.28	9±0.34	5±0.78	13±0.29	7.9±0.19	12±0.21	43
	1% EMS (in direct sowing)	21.0±2.49	6±0.49	8±0.23	14±0.53	6.0±0.12	8±0.13	54
	12 hr (*in vitro*)	22.0±1.13	4±0.70	9±0.26	16±0.56	8.0±0.17	9±0.14	51
M_3	Control (in direct sowing)	49.2±0.13	8±0.17	—	10±0.19	6.0±0.34	8±0.16	62
	(*in vitro*)	48.0±0.26	8±0.24	—	9±0.14	6.2±0.29	8±0.38	61
	0.25%EMS (in direct sowing)	26.8±0.34	7±0.18	—	9±0.20	7.0±0.15	9±0.17	50
	24 hr (*in vitro*)	38.3±0.25	7±0.26	—	12±0.27	8.0±0.19	11±0.16	50
	0.5%EMS (in direct sowing)	33.0±0.78	7±0.22	—	12±0.44	7.0±0.19	11±0.18	43
	24 hr (*in vitro*)	35.0±0.78	7±0.18	8±0.27	14±0.32	9.0±0.16	11±0.24	45
	1%EMS (in direct sowing)	21.0±0.44	7±0.23	—	8±0.31	8.0±0.19	9±0.12	57
	12 hr (*in vitro*)	22.0±0.75	5±0.21	5±0.16	11±0.26	8.0±0.18	11±0.18	54

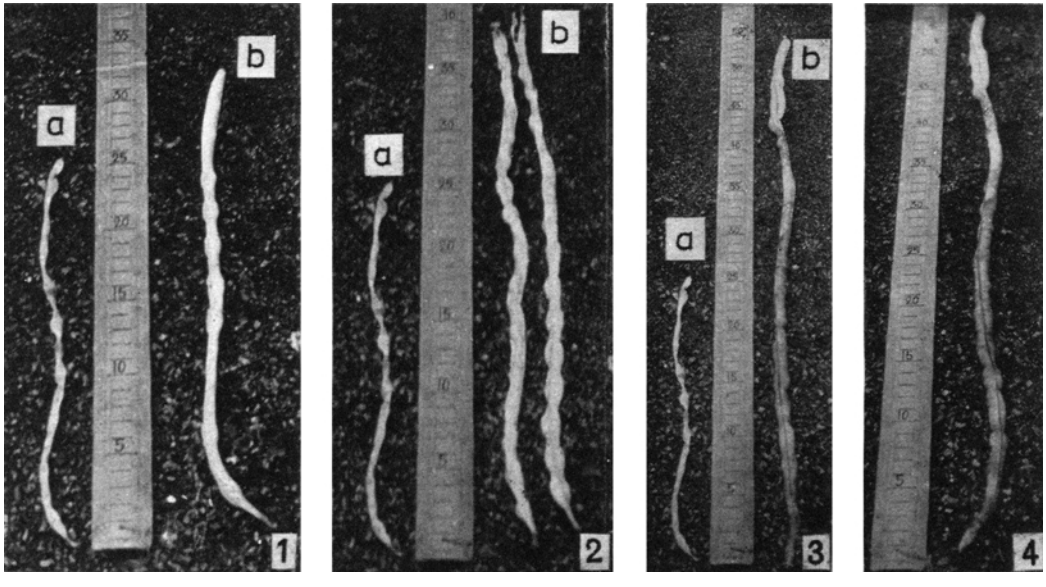
EMS for 12 hours. The mutants showed an increase in the number of nodes and leaflets. In *V. sinensis* the most important characters were increase in pod size and grain number per pod.

Pod length was nearly double that of the control in 0.5% and 1% EMS given for 24 hr and 12 hr respectively in M_2 as well as in M_3 generations. The number of seeds in elongated pods was also high, specially at 0.5% EMS given for 24 hours. A representative sample of the pods obtained in plants raised by direct sowing after EMS treatment (24 hr) as well as from plants raised from embryos cultured prior to transplantation are shown in figures 1–4. Pod length of up to 56 cm, bearing on an average 16 seeds (control with only 9 or 10 seeds) has been recorded. This long pod and high seed bearing mutant has been selected and stabilized (vide table 6–M_3–0.5% and 1% EMS–24 hr, 12 hr).

Though desirable mutants had been obtained both *in vitro* and in direct sowing, in general the frequency of desirable characters of such mutants had been found to be quite high in embryos grown *in vitro* prior to transplantation.

(iv) Other mutant characters of special importance

An important mutation involved flowering time. The controls take 60–62 days to flower. With EMS treatment one mutant, followed even up to the M_3 generation, showed flowering in *Vigna radiata* after 43–45 days and in *Vigna sinensis* after 40–41 days. Plants passing through *in vitro* phase and those developing from directly sown seeds showed the same frequency with regard to this character (tables 5–6).



Figures 1–4 Pods of *Vigna sinensis* var. black. *1a*, Control; *1b*, treated 24 hr with 0.5% EMS (direct). *2a*, Control; *2b*, treated 24 hr with 0.25% EMS (*in vitro*). *3a*, Control; *3b*, treated 24 hr with 0.5% EMS (*in vitro*). *4a*, treated 24 hr with 0.5% EMS (*in vitro*)

Table 6 *Morphological characters obtained from direct sowing and after* in vitro *growth in M_2 and M_3 plants of* Vigna sinensis

Generation	Treatment		Height	No. of pods	No. of leaflets	Pod size	No. of grains per pod	Days to flower
M_2	Control	(in direct sowing)	90.3±0.24	5±0.24	12±0.25	29.3±0.39	9±0.23	58
		(*in vitro*)	91.1±0.22	6±0.28	12±0.25	28.9±0.21	9±0.19	58
	0.25%EMS 12 hr	(in direct sowing)	98.0±0.79	10±0.46	31±0.51	31.0±0.28	15±0.10	49
		(*in vitro*)	99.0±0.73	10±0.40	29±0.64	34.0±0.39	16±0.35	48
	0.25%EMS 24 hr	(in direct sowing)	64.0±1.00	18±0.23	35±0.58	27.2±0.46	11±0.16	43
		(*in vitro*)	77.0±1.11	19±0.23	34±0.42	29.8±0.52	10±0.37	44
	0.5% EMS 12 hr	(in direct sowing)	68.0±0.72	12±0.32	24±0.44	31.4±0.89	10±0.53	46
		(*in vitro*)	74.0±0.83	15±0.30	31±0.45	32.9±0.74	10±0.56	46
	0.5%EMS 24 hr	(in direct sowing)	69.0±1.17	11±0.40	21±0.55	49.4±0.74	15±0.56	41
		(*in vitro*)	64.0±0.80	15±0.26	26±0.52	53.2±1.08	16±0.44	41
	1% EMS 12 hr	(in direct sowing)	69.0±0.28	9±0.29	24±0.46	51.6±0.35	11±0.27	40
		(*in vitro*)	64.0±0.64	16±0.34	20±0.45	56.0±0.92	12±0.12	40
M_3	Control	(in direct sowing)	98.4±0.18	4±1.00	11±1.51	28.2±0.20	9±0.21	58
		(*in vitro*)	93.2±0.42	6±0.24	12±0.26	28.3±0.20	9±0.25	58
	0.25%EMS 12 hr	(in direct sowing)	96.4±0.98	13±0.71	28±0.52	29.3±0.44	12±0.42	50
		(*in vitro*)	97.8±0.82	11±0.35	27.6±0.58	29.6±0.23 32.1±2.52	14±1.05	51
	0.25%EMS 24 hr	(in direct sowing)	58.4±1.13	18±0.21	33±0.63	25.9±0.52	9±0.36	42
		(*in vitro*)	95.8±0.85	19±0.32	27±0.55	29.8±1.06	11±0.40	43
	0.5%EMS 12 hr	(in direct sowing)	64.2±0.62	12±0.41	21±0.36	30.8±0.97	11±0.56	44
		(*in vitro*)	77.9±0.57	15±0.31	30±0.35	33.6±0.97	12±0.63	44
	0.5% EMS 24 hr	(in direct sowing)	60.6±1.09	11±0.46	24±0.80	46.7±0.83	14±0.40	42
		(*in vitro*)	67.9±0.87	15±0.36	26±0.56	52.1±1.08	16±0.44	43
	1%EMS 12 hr	(in direct sowing)	64.3±1.20	13±1.60	21±0.62	44.2±1.82	9±0.68	41
		(*in vitro*)	61.1±3.05	12±0.39	28±0.54	53.4±0.39	11±0.96	41

A petal mutant (crumpled mutant) in which the sepals were small and the petasl were incurved enclosing the stamens was noted. This character seems to follow a simple mutagenic inheritance. Such monogenic inheritance recessive to the wild type, had also been obtained by Appa Rao and Reddy (1975) in *Phaseolus mungo* and cowpea, in which the mutant gene exhibited pleiotropy, simultaneously affecting the shape, size and structure of the petals. The gene symbol crpt/crpt was proposed by Appa Rao and Jana (1976) for this allelic pair. In the present investigation also such a mutant was obtained in *Vigna sinensis.*

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