IRRADIATION OF DEVELOPING EMBRYOS — A TECHNIQUE FOR INDUCED MUTAGENESIS IN RICE

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In vitro dissected mature- and *in vivo* mature-, immature- and pro-embryos were irradiated with different doses of X-rays. Observations on chlorophyll- and macro- mutations including morphological-, agronomic- and sterile-types, as recorded from M_2 and M_3 generations have been presented. Variations induced due to micromutations of the 'plant height' character have been studied by calculating C. V. values and applying 't'-test of the data recorded in M_3-generation. Comparison of induced variations have also been made between the different doses of X-rays' treatments.

The chlorophyll- and macro-mutation spectra were widest in case of *in vitro* mature dissected embryos. The spectra narrowed down progressively with the relative immaturity of the embryos. Notable among the macro-mutants were the 'indica'-types. In M_3 generation the mean 'plant height' shifted both to negative and to positive directions but in different degrees. The negative shifting of mean occurred in more number of treatments than the positive. Significant differences between many individual treatments with respect to induced polygenic variations of plant height character have been observed.

INTRODUCTION

The earliest work on induced mutation in rice dates back to 1934 (Ichijima, K.). Works done on mutation in rice have been reviewed by Nayer (1965) and Gustafsson and Gadd (1966). From the literature available it appears that only a small amount of work on irradiating developing rice embryos, *in vivo* or *in vitro*, has so far been done (Chang and Mericle 1964). Induced mutations, using dissected mature and immature embryos have been reported in barley by Mericle *et al.* (1957, 1961, 1962, 1969), by Devereux *et al.* (1964) in *Nicottiana tabacum* L., and by Verma, Casper and Singleton (1962, 1963) and Chatterjee, Casper and Singleton (1965) in maize. Kawai (1962) and Kawai and Inosita (1965) have also studied in some detail the radiosensitivity, following gamma ray irradiation at definite developmental stages of rice plants.

Earlier in this laboratory mature and immature rice embryos of a few varieties of rice were irradiated with X-rays to study their radiosensitivity and mutation spectra (Bhaduri and Shom 1969; Bairagi 1968).

MATERIALS AND METHODS

Tainan-3, a photoinsensitive variety of *Oryza sativa* L. was used for X-irradiation. In the control sets, embryos from dehusked, soaked kernels were dissected and cultured in modified White's plant tissue culture medium. These embryos were X-irradiated from a 150 kv, 15 ma. X-ray machine. The X-ray doses administered were 10, 15, 20, 25, and 30 Krads. In the other set of experiments, intact rice (*O. sativa* L.) panicles were X-irradiated at definite intervals of time exposing the

developing proembryos to X-rays *in vivo*. The doses used varied from 2.5 Krads to 20.0 Krads and the ages of the materials (*in vivo* pro, early differentiating, immature- and mature- embryos) ranged from 24 hr to 15 days from the time and date of anthesis. All X-irradiated caryopses were allowed to attain 25 days age *in situ* and embryos from fully developed caryopses were cultured following the usual procedure. The seedlings were raised in tube culture and subsequently transplanted carefully to pots. They were reared to maturity in a net house and duly harvested.

In the following season (1968), M_2 rows of 50 plants each were raised in the field by sowing seeds from individual M_1 plants (X-irradiated generation). The chlorophyll and morphological mutations (macro-mutations) were scored, their frequencies and percentages were noted.

Two sets of M_3 progenies from M_2 seeds were raised later on. In one set, progenies of the lines segregating for mutations and the progenies of individual mutant plants (scored in M_2) were raised; in the other set, seeds from 50 randomly selected M_2 plants belonging to rows not segregating for macromutations were bulked for each X-ray dose of individual treatments (i.e., pro-, immature- and mature- embryos) The latter set also included the control treatment (i.e. dissected mature embroys X-rayed *in vitro*). Randomized block design with three replications was used for raising the bulk M_3 population. A replication consisted of 23 plots (each plot representing an X-ray dose of different treatments, the irradiated control plus one unirradiated set). Each plot consisted of ten rows with ten plants in each row. Row to row distance of 30 cm and plant to plant distance of 15 cm were maintained. Records of such quantitative characters like plant height, age at flowering, tiller numbers, panicle length, spikelet numbers, grain number— per panicle and per plant—were made from 40 plants selected at random from each plot (representing a dose) of each replication. The border rows and border plants were excluded.

To analyse the data on induced variation of quantitative characters the standard deviation, variance and coefficient of variation were calculated. The 't' test of mean plant heights was done to determine the significance of induced phenotypic variation of the treatments compared to each others including the unirradiated control.

From all quantitative characters studied, the results obtained on induced variation in respect of 'plant height' only have been presented.

RESULTS

Lethality—The X-ray induced lethality for different doses, as well as the calculated Ld_{50} values, are presented in Table I. Lower doses induced 50 and 100 per cent lethality with relative immaturity of the embryos. In case of the control, i.e. the mature embryos, seedling death commenced from germination onward. Eighty to eighty-five per cent of total seedling death occurred before they attained 10 days age. In case of proembryo treatments (at 24, 48 and 72 hr age) the lethality was more due to failure in kernel development or unsuccessful germination of seedlings. The pattern of lethality in case of 15 days old embryos *in vivo* was comparable to irradiated controls (i.e. *in vitro* mature embryos), both in respect of dosimetry as well as in respect of lethality percentage. In terms of actual percentage

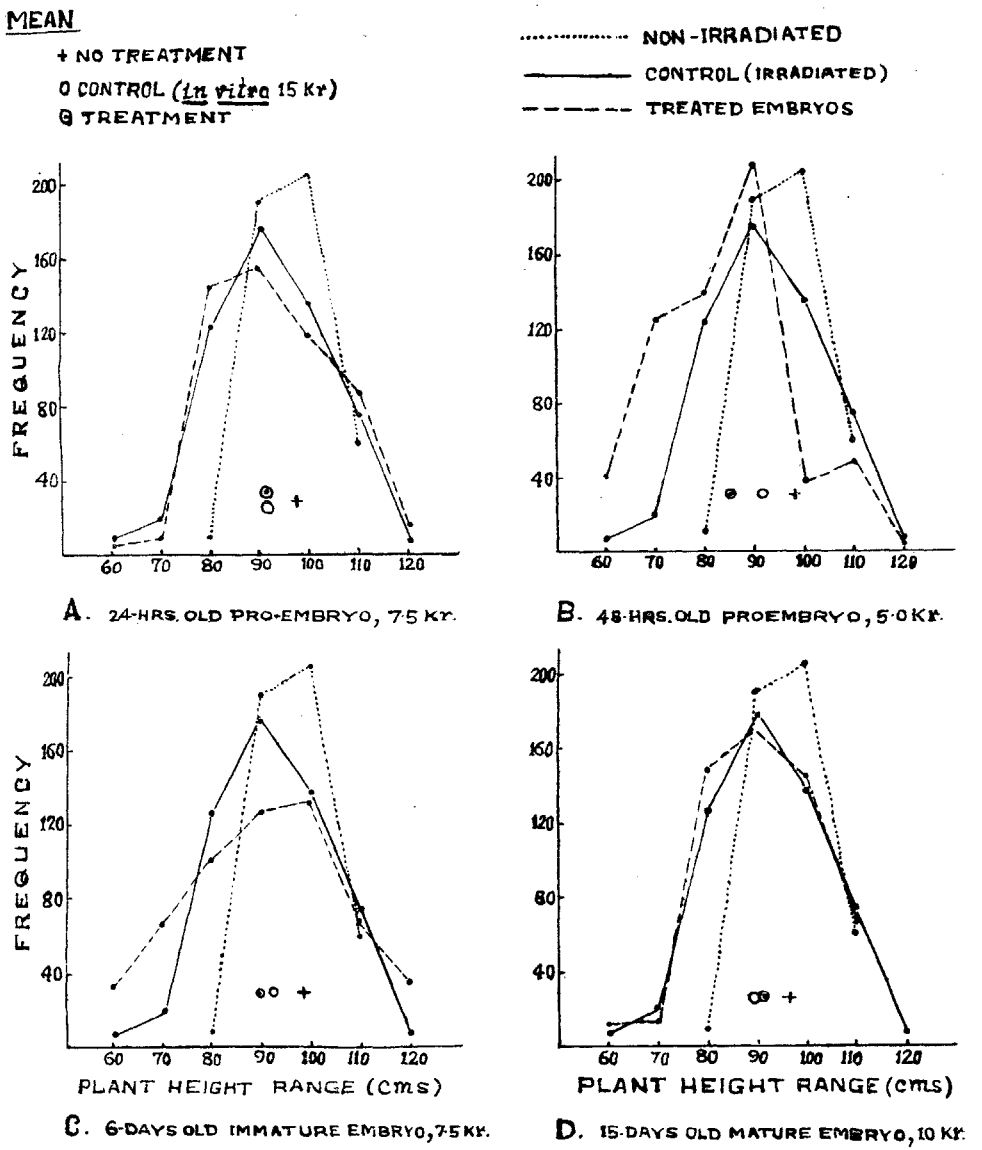

FIG. 1. Frequency distribution curves for quantitative character (Plant height in M_3).

of lethality and in manifestation of symptoms indicating the approach of death due to X-irradiations, the 6-days old immature embryos responded between the above two extremes. Production of some fully developed kernels, about 12 per cent, without any organised dissectable and culturable embryos was restricted to 6 days old irradiated kernels.

TABLE I

X-rays' induced lethality at different doses

(Material : *O sativa.* var. Tainan-3; mature-, immature- and proembryos)

Treatment	X-ray dose (Krads)	No. of embryos/ spikelets treated	No. of surviving plants at 30 days age	No. of surviving plants at Harvest	Ld_{50} (calculated Krad)
In vitro					
Mature	10.0	50 embryos	46	45	16.18
embryo	12.5	,,	38	34	
	15.0	,,	26	26	
	20.0	,,	18	16	
	25.0	,,	3	2	
In vivo					
Mature embryo	5.0	,,	42	42	
(15 days old)	7.5	,,	38	35	10.92
	10.0	,,	30	30	
	12.5	,,	21	20	
	15.0	,,	14	12	
Immature	2.5	60 spikelets	47	45	
embryo	5.0	,,	32	32	7.08
(6 days old)	7.5	,,	25	23	
	10.0	,,	17	16	
Proembryo	2.5	,,	29	29	
(72 hr old)	5.0	,,	26	25	5.00
	7.5	,,	14	14	
	10.0	,,	10	8	
	12.5	,,	—	—	
Proembryo	2.5	,,	29	28	
(48 hr old)	5.0	,,	23	23	4.85
	7.5	,,	11	8	
	10.0	,,	—	—	
	12.5	,,	—	—	
Proembryo	2.5	,,	26	23	
(24 hr old)	5.0	,,	20	19	3.52
	7.5	,,	13	13	
	10.0	,,	—	—	
	12.5	,,	—	—	

FREQUENCY AND SPECTRUM OF MACROMUTATIONS

The mutations scored in M_2 and M_3 generations have been classified under three heads, viz: (*a*) chlorophyll mutations, (*b*) morphological mutations, and (*c*) agronomic mutations.

Chlorophyll mutations, in case of irradiated controls as well as in the *in vivo* irradiated mature embryos occurred in heighest frequency (11.3 and 10.4 per cent respectively) in the M_2 and M_3 generations. The mutatants induced were albina, 70 per cent; chloroxantha, 21 per cent; chlorina, 7 per cent; alboviridis, 1 per cent and striata, 1 per cent of the total chlorophyll mutations. In case of the *in vivo* mature embryos the percentages of these types were 38, 26, 32, 4 and nil respectively. For immature embryos (X-rayed at 6-days age) the frequency of chlorophyll mutations was remarkably low. In case of 72-hr old and 48-hr old proembryos the total percentages of mutations were 3.5 and 2.7 per cent respectively. Proembryo treatment at 24 hr age did not produce any chlorophyll mutations.

The morphological and agronomic 'Macromutations' isolated in M_2 and M_3 generations included (*i*) profusely tillered dwarfs with 60–40 per cent reduction in height, (*ii*) rolled leaf, (*iii*) compact panicled, (*iv*) long, empty glumed, (*v*) sterile and semisterile plants, (*vi*) long and easy shattering-grained, (*vii*) tall and lighter green plants ('indica'-type), (*viii*) lax panicled, (*ix*) short panicled and (*x*) small grained types (Table II).

The macromutations and their occurrence, as calculated per 100 M_2 plants per dose of X-rays, have been presented in Table II. When the mutation spectra produced by X-irradiating at different stages of embryogeny were compared, it was observed that treatments of mature dissected embryos (control), *in vivo* 15 days old embryos and 6-days old immature embryos produced wider mutation spectra as compared to those obtained from proembryo treatments (24, 48 and 72 hr old). Although produced in a lower frequency, a number of agronomic types of mutations could be scored from the pro-embryo treatments (Table II).

Induced Variations due to Micromutations

Out of six set of quantitative characters studied, analysis of only one, i.e., 'Plant height' has been presented in this paper. Results presented in Tables III and IV indicate that the mean plant height from all treatments excepting serial numbers D, I, J, N, R, and U varied significantly from that of the unirradiated control. This is due to significant shifting of the mean either to positive or to the negative directions. The height was reduced in fourteen out of sixteen treatments. In case of treatments I, J and N there were neither significant shift in the mean, nor any change in the C.V. values as compared to that of control. In case of treatments like A, B, C, E, J, S and U, there were different degrees of reduction in mean plant height accompanied by increased C.V. (cf, Tables III and IV). However, the C.V. for mean plant height of the treatments D, J, N and U did not vary significantly from that of the unirradiated control in spite of reduction in mean plant height. The mean plant height was increased in the treatments 'K' and 'O' significantly as compared to the control. While the C.V. value was increased in the treatment 'K', it was reduced in the treatment 'O'.

It would appear from the Table IV that the mean values belonging to 6-days old pro-embryo treatments (J to M) have shown significant difference from that of the others in largest number of cases. The mean values of other pro-embryo treatments have also shown higher number of significant variations when compared to the

TABLE II

Macromutation types, percentages and mutation spectra scored by applying X-rays to embryos in vitro *and* in vivo *at different ages*

Treatment	State of material	X-ray dose (Krad)	Types of macromutations and their percentages (% families segregating in M_2)	Total % of families segregating for mutations in M_2 (Mature- immature-, proembryos)
A		10.0	1 stgr (1. 1.), d(0.4)	10.23
B		12.5	—	
C	*In vitro*	15.0	d (1.4), cp (0.6), rl (0.1),	
	Mature embryo		st (0.1), 'ind' (0.03)	
D		20.0	St (2.8), rl(1.9), sgr(1.8)	
E	*In vivo*	5.0	rl (0.8)	6.0
F	Mature embryo	7.5	—	
G	(15 days old)	10.0	Sm st (1.6), 'ind' (0.9), Sgr (0.2)	
H		12.5	d (0.2)	
I		15.0	rl (1.0), 1 stgr (1.0), cp(0.2), st(0.1)	
J	*In vivo*	2.5	sm st (1.7)	6.6
K	Immature embryo	5.0	st (1.3), d(0.8), sp(0.8)	
L	(6 days old)	7.5	—	
M		10.0	rl(1.0), 1 egl(1.0)	
N	*In vivo*	2.5	—	1.6
O	Pro-embryo	5.0	—	
P	(72 hrs. old)	7.5	—	
Q		10.0	d(1.0), sp(0.5), cp(0.1)	
R	*In vivo*	2.5	ind(0.2). 1 egl(0.2)	0.4
S	Pro-embryo	5.0	—	
T	(48 hrs. old)	7.5	—	
U	*In vivo*	2.5	1 egl(0.7)	0.9
	Pro-embryo	5.0	'ind' (0.2)	
V	(24 hrs. old)			
W	Control	Unirradiated	—	—

Types of mutations : cp—compact panicle; *d*—dwarf; *'ind'*—'indica' type tall, light green coloured plant; *l egl*—long, empty glume; *l stgr*—long, easy shattering grain; *rl*—rolled leaf; *Sgr*—small grain; *sm st*—semi sterile; *sp*—short panicle; *st*—sterile.

remaining treatments. Instances of such significant deviations in case of mature embryos (irradiated *in vitro* and *in vivo*) as well as the youngest pro-embryos' (24-hours old) treatments were fewer.

TABLE III

Means, coefficients of variation (C.V.) and 't'—test-values of X-ray irradiated populations and unirradiated control (Character—'Plant height')

Treatment serial	State of the material	X-ray doses (Krad)	Mean plant height (cm)	C.V.	't'
A	*In vitro* mature embryo	10 0	85.02±1.1	12.28	**
B		12.5	90.64±1.3	14.12	**
C		15.0	90.5 ±2.2	23.9	**
D		20.0	97.73±1.0	10.94	—
E	*In vivo* Mature embryo (15 days old)	5.0	86.16±1.2	11.72	*
F		7.5	97.02±1.6	15.50	**
G		10.0	92.12±0.8	8.35	*
H		12.5	98.46±2.2	22.44	**
I		15.0	98.9 ±1.0	10.41	—
J	*In vivo* Immature embryo (6 days old)	2.5	96.26±1.0	11.30	—
K		5.0	100.3±1.5	15.75	**
L		7.5	88.5 ±0.7	8.18	**
M		10.0	96.14±0.9	9.15	*
N	*In vivo* Proembryo (72 hrs. old)	2.5	95.02±1.0	10.20	—
O		5.0	100.15±0.6	6.03	**
P		7.5	92.4 ±0.6	7.14	*
Q		10.0	90.88±1.6	17.38	**
R	*In vivo* Proembryo (48 hrs. old)	2.5	98.59±1.1	11.36	—
S		5.0	86.12±1.5	16.89	**
T		7.5	93.67±0.8	9.07	**
U	*In vivo* Proembryo (24 hrs. old)	2.5	95.37±0.2	10.40	—
V		5.0	90.32±1.0	6.01	**
W	Control	Unirradiated	98.47±1.0	10.35	

't'—values : — insignificant; * significant at 5% level; ** significant at 1% level.

TABLE IV

't' test of mean values between individual treatments for the character *'Plant height' (cm)*

(A — W : Treatment serials as in the Table III)

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W
A		**	**	*	**	—	**	**	**	**	**	**	—	—	**	**	**	**	**	—	**	—	**
B			—	—	**	—	*	—	*	**	—	**	*	**	*	**	—	—	—	—	—	—	**
C				—	**	*	*	—	*	**	**	**	**	*	*	**	—	—	—	*	—	—	**
D					*	—	—	—	**	*	—	*	—	—	—	**	—	—	—	—	**	—	*
E						**	—	**	—	—	**	—	**	—	—	—	**	**	**	**	**	*	**
F							**	**	**	**	*	**	—	**	**	**	**	—	*	—	**	—	**
G								—	—	—	**	—	**	**	—	—	*	*	*	**	**	—	**
H									—	**	—	**	**	*	—	**	—	—	—	**	**	—	**
I										—	*	—	**	—	—	—	*	—	*	**	—	—	—
J											**	—	**	—	—	—	**	**	**	**	—	*	—
K												**	*	**	*	**	—	—	**	*	**	—	**
L													**	—	*	—	**	**	**	**	—	**	—
M														**	**	**	**	*	**	—	**	—	**
N															—	**	**	*	**	**	—	—	—
O																**	**	—	*	**	—	**	*
P																	**	**	**	**	—	—	—
Q																		—	—	**	**	—	**
R																			—	—	**	—	**
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't' values: — insignificant; *significant at 5% level; **significant at 1% level

Discussion

Lethality—It was observed from the calculated Ld_{50} values obtained from different treatments that there was an increase in radiosensitivity with relative immaturity of the embryos. These observations were in conformity with the findings of Mericle and Mericle (1957, 1961, 1962) in barley and Devraux *et al.* (1964) in *Nicotiana tabacum* L. For mature dissected embryos i.e., the control (treatments A to D), there was uniform germination irrespective of doses as reported earlier by Bhaduri and Shom (1969). With higher doses, germination was followed by death within a very short time i.e. 2 to 10 days after germination.

On the assumption that full expansion of coleoptyle and its emergence from embryo is the sign of effective germination, there was 97 per cent germination even at the highest doses (i.e. at doses inducing 90–100 per cent lethality at maturity). As is well known a large number of workers on seed irradiation have reported, however, various percentages of germination failure following irradiation with high doses of X-rays. The present observation of uniform germination of irradiated embryos may be attributed to (*a*) the aseptic cultural condition providing at the same time optimum hydration, temperature, light etc.; (*b*) supply of readily assimilable carbohydrate in the form of sucrose (this enables the embryos even with drastically impaired enzymatic system to thrive for some time); (*c*) absence of injurious effects of radiation-affected endosperm; and (*d*) the absence of mechanical interference of the seed coats limiting coleoptyle emergence.

While considering lethality of young proembryos (24 and 48 hr old) it was apparent that high X-ray doses induced more of embryo abortions and failure of kernel development. Almost all well formed seeds contained embryos which germinated and grew normally. Mericle *et al.* (1969), however, reported earlier that treated barley proembryos had led to appreciable reduction in seedling growth rate. The unaffected seedling growth of rice, observed during the present study was therefore at variance with that of Mericle *et al.* (1969) on barley. It appears that in case of rice the course of embryo differentiation and maturity, along with the endosperm development, have actually acted as a diplontic selection sieve allowing only those irradiated-embryo-bearing kernels to attain maturity which could produce viable seedlings without much of their vigours being lost.

The 6-day old embryo treatments produced between 17–23 per cent of fully grown but sterile kernels i.e. those bearing no properly differentiated dissectable and culturable embryos (treatments J to M). These could be due to cessation of embryo growth and probably failure in differentiation caused by X-rays' damage. In such seeds, however, starch filling and growth of endosperm were not seriously affected. Also, from the same treatments mature embryos were produced which on culturing could germinate but died subsequently due to X-ray induced organogenetic defects in them. From these observations on 6-days old embryo treatments it appears that in Tainan-3 at this particular age of seeds, the development of embryos and endosperms are not strictly dependant on each other. Because embryo death at this stage does not necessarily induce simultaneous death of endosperm.

MACRO-MUTATION SPECTRUM

Cholorophyll mutations—The occurrence of many types and large number of chlorophyll mutations in the treatments of *in vivo* and *in vitro* mature embryos was in conformity with most of the earlier observations. A sharp decline in the number of mutant types and their frequency in the treatments of 72-, 48- and 24-hr old pro-embryos may be attributed to a number of reasons. It appears that at the early phases of embryogenesis the genic attributes controlling the system of chloroplastid development as well as the prochloroplastids have a strong relationship with the survival potentiality of proembryos. It may be that by a process of diplontic elimination only the proembryos unaffected with respect to chloroplastids have differentiated and attained maturity. Conversely, it is also probable, that at these stages of embryogenesis the genic system controlling chloroplastid development may be highly resistant to radiation. Therefore chlorophyll mutuations, even at relatively high doses, are not produced. The occurrence of contrasting mutation spectra under such varied treatments i.e. mature embryos against early proembryos calls for further detailed investigation to (i) get an insight into the mechanism involved at the cell organelles' level in relation to the production of chlorophyll mutations through physical and chemical mutagenesis; and (ii) determine the intervarietal and/or interspecific differences in this respect. This is important because generally it is assumed that there is always a direct positive relationship between the spectrum of chlorophyll mutations and other macromutations. However, the present observation (treatments R–W) has revealed that important mutants could be scored even from those treatments which have failed to produce chlorophyll mutations (treatments Q, R, U and V, Table II).

The morphological and agronomic mutations—From the Table II it would be apparent that a wide range of characters were affected. The dwarf mutations occurred in highest frequency and next in order appeared the rolled-leaf mutations. However, these two mutants were scored from treatments A to M. The occurrence of other mutations was evenly distributed in case of the other treatments. Though the total number of mutation cases was reduced in pro-embryo treatments, a higher percentage of agronomic mutations (e.g. change in grain size and shape, age at flowering, increased and reduced tillering, compact and lax panicles, etc.) was scored from them (Table III).

The 'indica' type mutants appeared only from the treatments 'C' (15 kr-*in vitro* mature embryo), G (10 kr-*in vivo* mature embryo), R and V (proembryos 48-hr-2.5 kr and 24- hr-5.0 kr). The contrasting indica characters of the mutants were long grains, easy shattering habit, laxness of panicles and lighter green leaves. Swaminathan and siddiq (1968) have also reported isolation of the 'indica' type mutants from the variety Taichung Native—1 through chemical and physical mutagenesis.

Considering the types of mutants observed per treatment, and the frqeuency of occurrence of each mutation cases, it is apparent that the spectra changed from a wider to a narrower one in the following order : Controls (*in vitro* mature embryo)-15 days old embryos-6 days old immature embryos-72 and 48 hr old proembryos-24 hr old proembryos.

The results indicated that irradiation of pro-embryos and embryos with different doses of X-rays have largely been effective to induce significant changes in the mean plant height. Though the mean height, in majority of cases, was reduced there were two instances of its increase (treatments K and O). This decrease in height in some and increase in others as well as the high C. V. of unirradiated control indicate heterozygosity of the genes governing plant height in Tainan-3, a semitall 'Japonica trace of indica' rice. As the treatments have reduced the height in a number of cases it may be inferred that the direction of the previous selection was more towards tallness; and the genes contributing towards tallness were affected by X-irradiation at different stages of embryo genesis.

Those treatments (G, L, T and V) in which there was a significant reduction in plant height accompanied by reduction in C. V. values, micro-mutations (negative) leading to more homozygosity of the alleles governing height were indicated. Conversely, the treatments with reduced mean plant height and increased C. V., indicate further heterogeneitiy of the population with a preponderance of dwarf plants. Treatment 'K' showing increased mean plant height and the C. V. indicate an increase in heterogeneity in the population. Treatment 'O' on the other hand, registered increased height but much reduced C. V. suggesting a tendency towards allelic homogeneity. This part of the observation, however, warrant further study in order to understand fully the mechanism involved in production of a tall population through micromutation.

The tests of inter-treatment difference in mean values (Table IV) indicate that majority of the treatments are effective in inducing significant deviation from the other. The present observation has brought out clearly the wide scope for using developing and dissected mature-embryo mutagenesis for inducing wide range of variation through micromutations and therefore provide a greater scope for selection of desirable mutations. It has further indicated that the selection of the initial material for inducing micromutations should be a very important consideration.

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