INDUCTION OF MUTATIONS IN CULTIVATED RICE BY CHEMICAL AND PHYSICAL AGENTS[1]

1. The effects of isopropyl methanesulfonate, diethyl sulfate, and gamma radiations on seed germination and seedling height injury

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Abstract

Indica rice, IR-8, and *japonica* rice, Chianung 242, were-treated with the chemicals isopropyl methanesulfonate and diethyl sulfate as well as gamma irradiation. Mutagen solutions were buffered at pH 3, 7, 9, and 11 levels. Isopropyl methanesulfonate did not induce significant seedling height injuries, but, a number of chlorophyll mutants were found at the M_2 seedling stage. Diethyl sulfate caused severe damage with mutagen solutions buffered at pH 3 and 7. Those buffered at pH 11 stimulated seedling growth. Gamma irradiation also gave significant damage to seedling growth; nevertheless, seed germination was little affected.

Introduction

Since Oehlker (1943) injected ethylurethane and potassium chloride into the flower buds of *Oenothera* and found chromosomal aberrations, chemicals have been used as mutagens as well as radiations to induce variations on the heredity of plants. Auerbach (1949) suggested that chemical mutagens might be used as a mean to study the nature of mutagens and the mutations induced. During the past few years new alkylating chemicals have been proved to be highly effective mutagenic agents in higher plants (Heslot, 1965; Kawai, 1969, and Povilaitis, 1969). Their results indicate that the use of these agents yields a much wider range of genetic variability. The variability induced artificially is as useful as those of spontaneous origin (Nilan, 1967), but the rates of in-

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duced mutations can be greatly increased through the control of environmental factors (Konzak *et al.*, 1961). The new variability furnishes the raw material to study the alternate state of gene alleles and thus may provide into the structure of a gene. The induced variability will also increase the present gene pool and make possible the improvement of the material by genetic means for practical purposs. A good survey on the scccessful release of new crop varieties (rice, wheat, barley, soybean, and rape) which were improved by induced mutations was reported by Sigurbjörnsson (1968). These results indicate that the method of induced mutations can be used effectively in the field of plant breeding and will undoubtedly become more efficient with increased utilization. This first paper of the series reports our studies on the effects of isopropyl methanesulfonate (ipms) and diethyl sulfate (dES) as well as gamma irradiation treatments on rice germination and seedling growth.

Materials and Methods

Two rice varieties (Oryza sativa, $2n=24$) were used as the experimental materials. Variety Chianung 242 (CN242) is a Taiwan *japonica* variety. It has tall culms, large panicles, few tillers, vigorous growth, and late maturity. The other variety IR-8 is an *indica* variety from the International Rice Research Institute, the Philippines. It carries a semi-dwarf gene, Dee-geo-wu-gin. It has abundant tillers, long-kernels, and also late maturity. Seeds of those two varieties were stored in desiccators and their moisture content controlled to approximately 13% (Nilan and Konzak, 1961).

Seeds were pre-soaked in running water for 24 hours at room temperature and treated with buffered mutagen solutions. Buffers of glycine, potassium phosphate, and sodium carbonate were made at a concentration of 0.1 M, at pH levels of 3, 7, and 11. Before making the mutagen solution, the required volumes of buffers were measured into Erlenmeyer flasks and held in a temperature-regulate water bath for several hours to bring the solution down to the required temperature. Seeds were washed for 24 hours in running water after the treatments, germinated in a temperature-controlled room (27–28°C) for three days, and then grown in flats (300 seeds per treatment) in a greenhouse (temperature not controlled). Seedling height was measured when the control seedling reached approximately 20 cm. There were five experiments in this study. Details of the treatments for each experiment are given in table 1.

Experiment 1. Seeds of varieties CN242 and IR-8 were pretreated with ipms at 5°C for 6 hours after pre-soaking. This would give ample time for mutagen solutions to penetrate into the tissue. Mutagen solutions were prepared at concentrations of 0.03, 0.04, 0.05, and 0.06 M. Treatments were done at 24°C

Table 1. *List of treatments in different experiments*

Expt. No.	Name of var.	Mutagens	Concentration M	Presoak hrs.	Temp.	Pre-treatments		pH	Treatments		Postwash hrs.	Growth temp. C
						hrs.	temp. C		hrs.	temp. C		
1	IR-8*	ipms	0.03; 0.04; 0.05; 0.06	24	22-24	6	5-6	9	3	24	24	25-32
	CN242**	ipms	0.03; 0.04; 0.05; 0.06	24	22-24	6	5-6	9	3	24	24	25-32
2	IR-8	dES	0.01	24	22-24	—	—	9	24, 48, 72, 96 120, 144	10	24	28-34
3	CN242	dES	0.01	24	24-30	—	—	3, 7, 11	72, 96, 120, 144, 168	10	24	25-32
	CN242	dES	0.01	24	15-22	—	—	3, 7, 11	72, 96, 120, 144, 168	10	24	10-22
	IR-8	dES	0.01	44	18-25	—	—	3, 7, 11	72, 96, 120, 144, 168	10	24	15-25
4	CN242	dES	0.01	24	18-25	—	—	3, 7, 11	24, 48, 72, 96. 120	10	24	18-25
5	CN242 (H_2O, 13%)	gamma ray							20, 25, 30, 40 50 (kr.)			20-25

* *indica;* ** *japonica*

for three hours after the pre-treatments. Solutions of mutagen were not changed in this experiment.

Experiment 2. Agent dES was used to treat variety IR-8. Treatments were done at 10°C for 24 to 144 hours. Mutagen solutions were buffered at pH 9 and changed at each half-life time of every 12 hours.

Experiment 3. Variety CN242 was treated with dES. The mutagen solutions were buffered at pH 3, 7, and 11. Treatments were from 72 to 168 hours. Seedlings were grown in July and December, 1969.

Experiment 4. The treatments of experiment 3 were repeated. However, the treatments were from 24 to 120 hours instead of 72 to 168 hours. Seedlings were grown in March, 1970.

Experiment 5. Gamma radiation was used in this investigation to treat variety CN242. Doses of 20, 25, 30, 40, and 50 kr were applied. Seeds were hydrolyzed within three hours after the irradiation.

Formula for non-linear regression analysis used is given in follows:

$$W=\frac{A}{(1+be^{-kt})^{\frac{-1}{1-m}}}$$

W=theoretical curve of response
A=maximm response (%)
k=response per day or per unit (%)
m=shape of response curve
b=basic response (with no biological significance)

Experimental Results

Experiment 1. In the 0.06M treatment, seedlings of variety CN242 were shorter than those of the untreated control. Data of the experiment are given in table 2. Statistical differences of 5% level among all treatments for variety

Table 2. *Comparison of CN242 and IR-8 seedling height (cm) treated with ipms grown at 25-32°C (Randomized complete block design with five replications)*

Variety \ Concentration / height	Control	0.03M	0.04M	0.05M	0.06M	F Value
CN242	23.87[1]	23.09	22.66	22.81	22.53	3.1823*
IR-8	21.42	21.39	21.54	21.41	21.23	0.4261

F Value (0.05%)=3.01
F Value (0.01%)=4.77
1) Each value was the mean of 60 plants.

CN242 were found by the analysis of variance, but no difference for variety IR-8. This indicates that the chemical ipms does not cause serious biological damage at the seedling stage. Nevertheless, the treatments given to both varieties were effective, and the effect had been seen at the M_2 seedling stage at which a great number of chlorophyll mutations were revealed.

Experiment 2. This involved the treatments of variety IR-8 with dES at low temperature (10°C) for a long period. This was done because dES has a short half-life and low temperature can slow down the hydrolytic rate. Data of the experiment are given in table 3. Seedlings from the treated seeds were generally shorter than those from the control, and the seedling height injuries (per cent shorter than the control) increased with the increase of treatment times. The percentage of the injuries related to the length of exposure to the mutagenic agent is given in figure 1. The injuries increased gradually from 24 to 72 hours, but rapidly from 72 to 96 hours. The theoretical curve was non-linear with the time of 73.2 hours required for the major part

Table 3. *Comparison of IR-8 seedling height treated with dES grown at 28-34°C.*

Treatment (hrs.)	Seedling height cm		Seedling height injury %	No. of seedlings calculated Cont. Tr.
	Cont.*	Tr.**		
24	22.58±0.32;	22.47±0.25	0.49	100 138;
48	19.78±0.22;	18.22±0.18	7.88	100 140;
72	20.95±0.22;	19.64±0.23	6.67	100 133;
96	20.49±0.22;	15.59±0.22	23.93	100 129;
120	19.02±0.28;	13.78±0.19	27.58	100 138;
144	19.15±0.27;	13.23±0.24	30.91	100 124;

* Control; ** Treated.

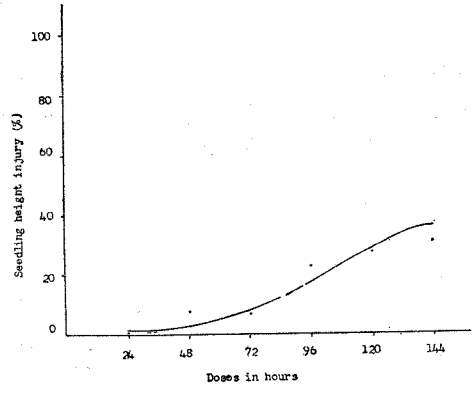

Figure 1. Comparison of IR-8 seedling height injury treated with dES.

of response. This indicates that the biological system could tolerated the chemical fairly well within 72 hours but not beyond that period. Variances of seedling height from treatment plots were generally greater than those from the controls.

Experiment 3. Results of the experiment are given in table 4, and the theoretical seedling height injuries are plotted in figure 2. These data show that

Table 4. *Comparison of CN242 seedling height treated with dES grown at 25–32°C.*

treatment No.	pH	treatment times (hrs.)	seedling height cm	seedling height injury %	seedling survival %*
Control	(6.2)	0	22.72±0.2	0	100
1	3	168	0 0	100.00	0
2	3	144	5.14±1.02	77.38	10
3	3	120	15.34±0.49	32.48	100
4	3	96	20.11±0.29	11.49	100
5	3	72	20.32±0.23	10.56	100
6	7	168	4.78±0.49	78.30	28.6
7	7	144	4.76±0.18	78.31	97.1
8	7	120	18.59±0.31	18.22	100
9	7	96	20.98±0.16	7.66	100
10	7	72	22.14±0.23	2.56	100
11	11	168	22.38±0.15	1.49	100
12	11	144	23.02±0.16	- 1.32	100
13	11	120	23.51±0.17	- 3.48	100
14	11	96	23.13±0.20	- 1.80	100
15	11	72	22.31±0.17	1.80	100

* 140 seeds were planted for each treatement.

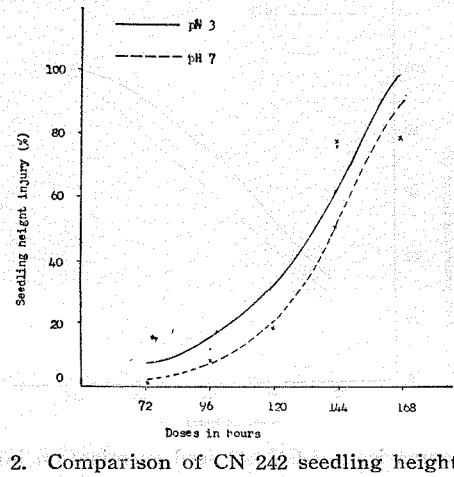

Figure 2. Comparison of CN 242 seedling height injury treated with dES grown at 25-32°C.

most seedlings from the treated seeds were shorter than those from the control. Mutagen solutions buffered at pH 3 and 7 gave more drastic damage to seedling height. The theoretical major response of injuries became significant after 121.2 hours treatment. On the other hand, mutagen buffered at pH

Table 5. *Comparison of CN242 seedling height treated with dES grown at 10–22°C.*

treatment No.	pH	treatment times (hrs.)	Ave. seedling height cm	seedling height injury %	germination * %
Control	6.2	0	17.30±0.23	0	93.66
1	3	168	9.50±1.19	45.09	3.33
2	3	144	8.33±0.70	51.85	5.67
3	3	120	8.83±0.46	48.96	19.33
4	3	96	9.10±0.33	47.40	46.00
5	3	72	10.37±0.35	40.06	62.67
6	7	168	9.97±0.73	42.37	11.67
7	7	144	11.10±0.86	35.84	12.33
8	7	120	11.23±0.42	35.09	36.33
9	7	96	11.93±0.36	31.04	53.67
10	7	72	12.70±0.33	26.59	70.00
11	11	168	14.65±0.43	15.32	39.33
12	11	144	13.63±0.36	21.22	71.30
13	11	120	14.07±0.29	18.68	73.00
14	11	69	13.49±0.29	22.03	82.33
15	11	72	14.19±0.38	17.98	82,67

* 300 seeds per plot.

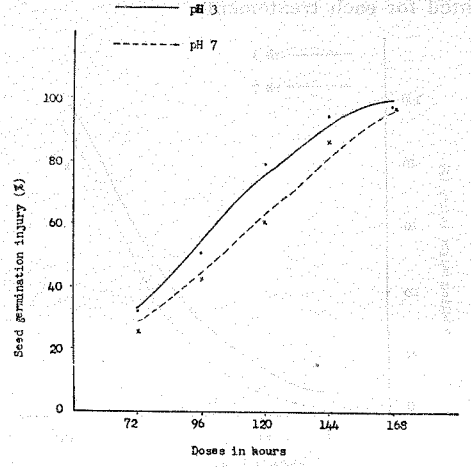

Figure 3. Comparison of CN 242 seed germination injury (%) treated with dES grown at 10-22°C.

11 not only gave no significant damage but actually stimulated germination. Thus, some increases in seedling height were found from 96 to 144 hours. Variances of seedling height injuries were larger at pH 3 and 7 than at pH 11. Seedling survivals decreased only in treatments of 144 and 168 hours at pH 3 and 7 levels. Plants from these two treatments grew slowy. Therefore, no

Table 6. *Comparison of of IR-8 seedling height treated with dES grown at 15–25°C.*

treatment No.	pH	treatment times hrs.	Ave. seedling height cm	seedling height injury %	germination * %
Control	6.2	0	14.15±0.13	0	97.33
1	3	168	9.00±0.30	36.40	42.67
2	3	144	9.73±0.22	31.24	64.33
3	3	120	11.21±0.18	20.78	72.00
4	3	96	11.79±0.16	16.68	84.67
5	3	72	12.18±0.19	13.92	90.09
6	7	168	11.54±0.13	18.45	73.67
7	7	144	12.74±0.12	9.96	84.33
8	7	120	12.96±0.15	8.40	82.33
9	7	96	13.48±0.15	4.73	93.00
10	7	72	13.52±0.11	4.45	95.00
11	11	168	13.52±0.12	4.45	90.33
12	11	144	14.67±0.12	- 3.67	88.67
13	11	120	14.65±0.14	- 3.53	91.67
14	11	96	14.30±0.10	- 1.06	95.00
15	11	72	14.87±0.13	- 5.09	93.00

* 300 seeds per plot.

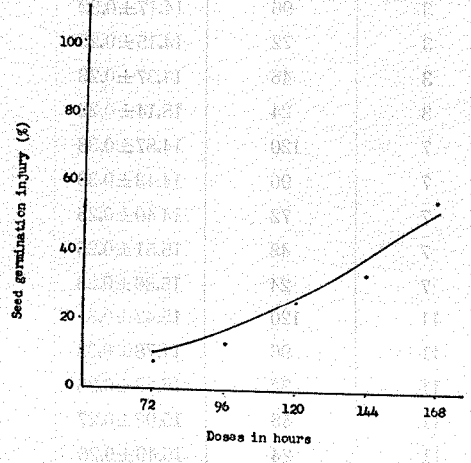

Figure 4. Comparison of IR-8 seed germination injury (%) treated with dES (pH 3) grown at 15-25°C.

plant ripened on time; no seeds were recovered in the second cropping season of 1969.

The same experiment was repeated in December, 1969. The temperature during the seedling stage was much lower than those in July. Seedlings from pH 3 solutions also carried the largest injuries, and those from pH 11, the least (table 5). The injuries were generally increased in this material compared with those which were done in July. However, unlike the former, the injuries did not increase as a steep slope. The variance of seedling height was larger among taller plant, while shorter seedlings varied less. The theoretical curves of seed germination injury at pH 3 and 7 are plotted in figure 3. Those buffered at pH 11 gave a linear increase of injuries with the increase of dose. The injury was most at pH 3 and least at pH 11.

Variety IR-8 was also used in the experiment. Data are given in table 6. Seedling height injuries also increased with dose of mutagen at pH 3 and 7, however, solutions buffered at pH 11 stimulated seedling growth. Negative seedling height injuries were found with treatments of 72, 96, 120, and 144 hours. Germination rate declined with the increase of dose. The theoretical curve of seed germination injury (pH 3) is given in figure 4. Those at pH 3 and 7 showed linear reaction. Treatments done at pH 3 were most effective

Table 7. *Comparison of CN242 seedling height treated with dES grown at 18-25°C.*

treatment No.	pH	treatment times hrs.	Ave. seedling height cm	seedling height injury %	germination * %
Control	6.2	0	17.80±0.38	0	95.66
1	3	120	13.28±0.49	25.40	16.33
2	3	96	14.17±0.32	20.40	32.67
3	3	72	14.15±0.23	20.50	74.33
4	3	48	14.37±0.23	19.27	90.67
5	3	24	15.14±0.22	14.95	96.67
6	7	120	14.87±0.38	16.46	32.00
7	7	96	14.43±0.30	18.93	49.33
8	7	72	14.40±0.25	19.11	68.67
9	7	48	15.51±0.25	12.87	89.00
10	7	24	15.86±0.23	10.90	94.33
11	11	120	15.42±0.31	13.37	89.67
12	11	96	14.78±0.33	16.97	92.67
13	11	72	15.23±0.26	14.44	93.33
14	11	48	15.02±0.27	15.62	92.67
15	11	24	15.49±0.26	12.98	93.00

* 300 seeds per plot.

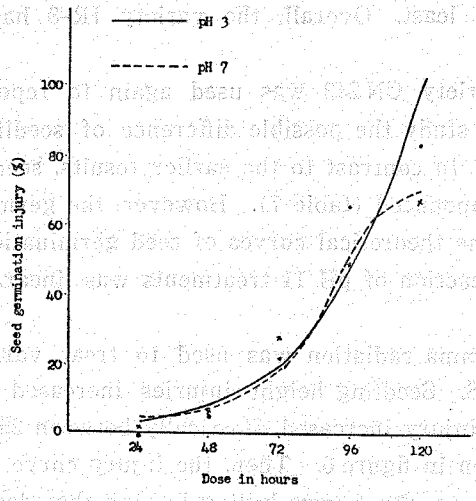

Figure 5. Comparison of CN242 seed germination injury (%) treated with dES grown at 18-25°C.

Table 8. *Effect of gamma radiations on seedling height injuries and germination rates (%) (Randomized complete block design with five replications)*

	Control	20 kr	25 kr	30 kr	40 kr	50 kr
Seedling height injuries	0	9.50	11.42	34.20	61.0	66.01
Germination rates	94.0	92.53	92.53	93.33	92.13	91.53

* 300 seeds per treatment
M. S. (Seedling height) =4,454.15**

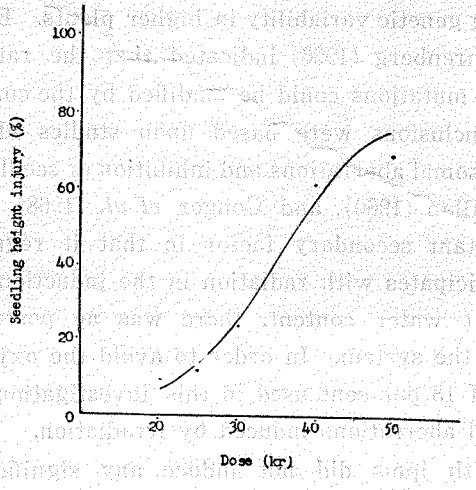

Figure 6. The effect of gamma radiation on CN242 seeds.

and those at pH 11 least. Overall, the variety IR-8 had less damage than CN242.

Experiment 4. Variety CN 242 was used again to repeat experiment 3 in march, 1970 and to study the possible difference of seedling growth between spring and winter. In contrast to the earlier results, seedling height injuries were much less pronounced (table 7). However, the germination rate differed with pH levels. The theoretical curves of seed germination injury are given in figure 5. The reaction of pH 11 treatments was linear, and was also least effective.

Experiment 5. Gamma radiation was used to treat variety CN242. Results are given in table 8. Seedling height injuries increased from 9.5 to 66 per cent. The slope of injury increased drastically between 25 and 40 kr. Theoretical curve is given in figure 6. Then, the injury curve flattened out after 40 kr. This was due to the severe killing beyond that dose. Germination was little affected with the increase of dose, however.

Discussion and Conclusion

Methods of induced mutation are known to have the advantage of affecting only a few of the total number of genes in the genome. Thus, it is highly desirable to apply this method on those varieties which carry a great number of promising characters, but only have a few weaknesses to be overcome. Varieties CN242 and IR-8 were used respectively to reduce their tall stature in order to increase its lodging resistance (Bozzini and Scaracia-Mugnozza, 1967) and to induce early maturation for better adaptibity to the multiple cropping system of Taiwan.

Treatment of seeds by mutagenic agents is one of the most important methods of inducing genetic variability in higher plants. Early studies of Caldecott (1954) and Ehrenberg (1956) indicated that the rates of production of irradiation-induced mutations could be modified by the control of seed moisture content. Their conclusions were based upon studies which determined frequencies of chromosomal aberrations and inhibition of seedling growth. However, recent studies of Nilan (1960), and Conger *et al.* (1968) revealed that water was only an important secondary factor in that it regulates the degree to which oxygen participates with radiation in the induction of biological effects. Above 11 per cent water content, there was no postradiation damage by bubbled oxygen to the system. In order to avoid the oxygen effect, the seed moisture content of 13 per cent used in this investigation should avoid most of the chromosomal aberrations induced by irradiation.

Treatments with ipms did not induce any significant seedling height injuries in this study. This may be because ipms has no significant degrad-

ation rates in the buffered solution of pH 9. This chemical was previously studied by Wickham *et al.* (1969) by means of infra-red spectrophotometry. They concluded that the ipms reacted by the S_N1 mechanism, and that the isopropyl group was less toxic to the biological system. A number of chlorophyll mutants, mostly albina, chlorina, and striata were found at the M_2 seedling stage, i. e., 6.2 to 11 per cent and 5.2 to 8.8 per cent on the M_1 plant basis from varieties CN242 and IR-8 respectively. The appearance of chlorophyll mutants in a frequency greater than that of the controls indicates that the mutagen did react with the germplasm of the seeds. Although the chemical did not immediately cause biological damage at the seedling stage, the interaction between the chemical and the genetic material existed and has been carried over to the next generation. The mutants can possibly be used as markers of mutagenic effectiveness, since the frequencies of morphological mutation are known to be proportional to the chlorophyll mutant frequencies (Doll and Sandfaer, 1969; Bozzini and Scarascia-Mugnozza, 1970).

Alkylating agents produce acid during hydrolysis thereby decreasing the pH of the treatment solution with resulting damaging side effects (Froese-Gertzen *et al.*, 1963). Konzak *et al.*, (1964) showed that the hydrolysis rate of ethyl methanesulfonate was not affected by low pH, but the biological system was rather sensitive to low pH. Hydrolysis of dES was highly dependent upon the influence of temperature. The half-life of the chemical was found to be 59.2 hours at 0 C but only 1.01 hour at 30 C (Konzak *et al.*, 1961). In order to lengthen the half-life of dES before it can react with the genetic material, treatments were done at low temperature (10 C). Under this condition, there was ample time for the mutagen solution to penetrate through the hulls. Rice seeds can be treated for a long period (24 to 168 hours) which is in contrast with the short treatments of a few hours treatments used on other crop plants. The seedling height injuries of pH 3 and 7 mutagen solutions followed logistic curves, while those at pH 11 are linear. The damage was severe at low temperature during growth. This suggested that the acidity has played a role in causing biological damage. The biological system can tolerate the treatment to certain extent before the damage becomes significantly apparent. Those buffered at pH 11 may not damage to the system, and the damage come from the reaction of the mutagen with the genetic material. Besides, the mutagen solutions at pH 11 stimulated seedling growth. These results were somewhat similar to those of Rao *et al.* (1965). They found that when the mutagen solution was buffered at pH 2 to 3, there were 16 to 18 per cent seedling injuries, whereas when the solution was buffered at pH 11 only 8.6 per cent injury was induced. The differences in injuries caused by different pH solutions were much more severe than those of Rao. They might be due

to the longer treatment times and the higher sensitivity of rice.

Gamma radiation gives a similar retardation to seedling growth. Rice tolerates the irradiation fairly well at low doses of 20 to 25 kr, then seedling height injuries increase drastically with the increase of dose (Woo, 1969). The major part of response was beyond 40 kr treatment. The germination rate, however, dose not seem to be affected as much by gamma radiation as by chemical mutagens. The decrease of M_1 field survivals and M_1 fertilities was more significantly observed in the radiation than in the chemical treatments. This indicates that the radiation treatments produce damage not only at the seedling stage but also to the adult plants. Chemical mutagen solutions buffered at high pH level can avoid most of the seedling damage, and the mutagenic effect can proceed successfully to subsequent generations through normal seedling growth and highly fertile M_1 plants.

水稻化學及物理誘變之研究

1. 種子發芽率及秧苗生長速度，受到 ipms, dES 及 gamma 線處理的影響

吳旭初　吳智明　戴依妹

應用化學誘變劑 ipms, dES 和 gamma 線，處理秈稻 IR-8 和粳稻嘉農 242 號兩品種，以研究該等誘變劑對種子發芽率，及生長速度的初步反應。化學誘變劑使用時係先溶解於 pH 3, 7 及 11 三種緩衝液中。ipms 對秧苗高度的影響不明顯，但是在 M_2 代却能出現大量的葉綠素變異體；dES 於 pH 3 和 7 兩種緩衝液中使用時，對發芽率及秧苗高度的影響顯著，但在 pH 11 緩衝液中使用時，反而能够促進秧苗生長的速度，由此可見 pH 度對稻種有顯著的作用。

Gamma 線對秧苗生長速度之影響亦極明顯，使用單位高者，生長緩慢，但是對發芽率的抑制作用不大。

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