Received: 29 Mar 2021 | Revised: 15 May 2021 | Accepted: 20 May 2021

Electronic Journal of Plant Breeding

Research Article

Assessment of mutagenic sensitivity in blackgram variety CO 6

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Abstract
The present study was conducted to examine the mutagenic effect of ^{60}Co gamma rays and a combination of ^{60}Co gamma rays with EMS in blackgram variety CO 6. Based on the LD_{50} dose, the seeds were treated with three different doses of gamma rays (200, 300, 400 Gy) and recurrent doses of 20 mM and 30 mM EMS were treated with gamma ray irradiated seeds for combination treatments. The M_1 generation was evaluated in the field for the following parameters *viz.,* germination percentage, plant survival percentage, plant height at 30 DAS and maturity, pollen fertility and seed fertility. A dose-dependent decrease was noticed for all the characters under this study in M_1 generation. Overall, the maximum reduction was noticed at a higher dose of 400 Gy gamma rays and 400 Gy + 30 mM EMS mutagenic treatments. Among the mutagenic treatments, a combination of ^{60}Co gamma ray + EMS exhibited the maximum effect with a higher percentage of reduction in all the traits while increased pollen sterility was found to be associated with a corresponding increase in dose/concentration of mutagenic agents. A significant positive correlation was observed between viable mutation frequency and biological damage caused by mutagens in M_1 generation. Hence, the higher doses of ^{60}Co gamma rays (300 and 400 Gy) and ^{60}Co gamma rays +EMS (300 Gy+ 30; 400 Gy+ 20 and 400 Gy+ 30 mM) proved to be efficient in increasing the mutation frequency towards desirable directions.

Key words
Blackgram, combination treatment (^{60}Co gamma rays + EMS), ^{60}Co gamma rays, mutation breeding.

INTRODUCTION

Blackgram is considered to be the most ancient and third important pulse crop due to its nutritional quality and suitability to the cropping system. It contains a rich source of proteins (25-26.20 %), 56.60 per cent of carbohydrates, and low fat (1.20%) content, besides minerals, vitamins and amino acids (Panhwar, 2005). It is known as “poor man’s meat” since it is contributing a major share of the dietary protein of the vegetarian population. They are widely grown in the Indian subcontinent followed by Thailand, Australia and other Asian and South Pacific countries, on a small scale (Harouna, 2020). India is the largest producer of blackgram contributing 30.59 l.t. annually from an area of about 56.02 l. ha, although, the average productivity is quite low with 546 kg/ha (Indian Statistical Data Base, 2019). In Tamil Nadu, it is cultivated in an area of 4.40 l, ha with the production and productivity of 2.74 l. t. and 622 kg /ha respectively (Indian Statistical Data Base, 2019).

The prime limiting factors for realizing the higher yield of this crop can be attributed to lack of genetic variability, indeterminate growth habit, canopy architecture, poor harvest index, cultivation in marginal lands and

susceptibility to pests and diseases (Azeem *et. al.,* 2019). It has been well demonstrated that the mutation breeding plays a major crucial role in plant breeding for improving various agri-horticultural crop cultivars with specific traits. Among the different mutagenic agents, gamma rays were routinely employed to produce a large number of mutants in blackgram (Souframanien and Pandey, 2006). The effect of induced variation relies on three important factors namely efficiency and effectiveness of mutagens, mutagenic doses and nature of plant material (Usharani and Kumar, 2015).

Large numbers of populations are preferred for evaluation as the induction and identification of a mutation in a specific gene is very difficult as it may be time consuming, costly, and increase complexity in selection. Alternatively, identifying effective mutants in M_1 generation and forwarding them to next generation reduced the above difficulties and may lead to the efficient selection of desired trait. With this background, the current investigation was carried out to determine mutagenic sensitivity and the extent of an effect caused by mutagen on the surviving plants. Besides this, correlation existing between M_1 mutagenic sensitivity factors and mutation frequency in M_2 generation was also studied.

MATERIALS AND METHODS

The dry seeds of black gram variety CO 6 were exposed to gamma rays from the Cobalt 60 (^{60}Co) gamma source in the gamma chamber installed at BARC (Bhabha Atomic Research Centre), Mumbai. Well-filled healthy seeds of CO 6 were treated with gamma rays at 200 Gy, 300 Gy, and 400 Gy based on the LD_{50} dose (Lethal dose 50) fixed by assessing germinability parameter under *in-vitro* condition. To evaluate the combined effect of ^{60}Co Gamma rays and EMS, three different doses of ^{60}Co gamma-irradiated seeds were subjected to 20 mM and 30 mM EMS (Ethyl Methane Sulfonate) as a recurrent treatment (200 Gy + 20 mM, 200 Gy + 30 mM, 300 Gy + 20 mM, 300 Gy + 30 mM, 400 Gy + 20 mM, 400 Gy + 30 mM) based on the LD_{50} dose following the procedure described previously with gamma rays.

A total of 3400 seeds from each treatment of ^{60}Co gamma rays and 750 seeds from a combination of ^{60}Co gamma rays with EMS treatments were sown along with non treated seeds as control (CO 6) using Randomized Complete Block Design (RCBD) with two replications in a spacing of 30 x 10 cm at the experimental plots of Department of Pulses, Tamil Nadu Agricultural University, Coimbatore. All the standard agronomic practices and plant protection measures were adopted.

In M_1 generation, the following parameters *viz.,* seed germination, survival of plant (30th day), plant height at 30th day, and maturity, pollen and seed fertility were recorded to evaluate the genetic response of blackgram CO 6 in relation to various doses of mutagens.

The germination percentage was measured by counting germinated seeds from the third to seventh day by keeping the emergence of a cotyledonary leaf as an indication. Germination percentage was worked out in each treatment separately using the following formula,

$$\text{Germination percentage (\%)} = \frac{\text{No. of germinated seeds}}{\text{Total no. of seeds}} \times 100$$

The number of plants that survived on the 30th day was counted and expressed in percentage. The height of the plant was measured from the ground level to the tip of the plant on the 30th day and at maturity. For conducting pollen fertility experiments, flowers were collected from 150 random plants of each treatment during early morning hours, along with control. Pollen grains were collected from freshly dehisced anthers and stained with 1% of potassium iodide solution. The observation was made under a compound light microscope at the magnification of 4X and the images were captured using Camera H1C and Scope image 9.0.1 software. The well filled and stained pollens were counted as fertile ones, whereas the unstained and shrunken were recorded as sterile pollens.

$$\text{Pollen fertility (\%)} = \frac{\text{No. of stained pollens}}{\text{Total no. of pollens observed}} \text{ X 100}$$

At the harvest stage, about 200 random plants were selected in each treatment. The number of well-filled and ill filled seeds per pod was counted. The mean seed fertility was calculated by the percentage of well-filled seeds to the total number of seeds for each selected plant in their respective treatments.

All the aforementioned parameters were recorded for each treatment and expressed as per cent over control and reduction. The percentage data on germination, pollen, seed fertility and survival were analyzed upon *arcsine* transformation (Hayes *et al*., 1955) and first-order statistical analysis was done using Statistical Package for the Social Sciences (SPSS) software version 20.0.

A total of 900 families (100 families/ treatment) of M_1 generation were forwarded to M_2 generation. The viable mutation frequency [deviation in general morphology of plants (plant height, shape and size of the leaf and pod respectively)] was calculated based on M_1 family (M_1F) and M_2 plant basis (M_2P) for individual treatment wise. Viable mutants were recorded in M_2 mutagenized populations from seedling to maturity stage of the crop. This parameter is used to identify the response of genotype in terms of morphology and various parts of plants affected towards mutagenic sensitivity.

The mutation frequency was calculated as follows,

Mutation frequency on M_1F basis =

$$\frac{\text{Number of M1 families segregating in M2 generation} * 100}{\text{Total number of families}}$$

Mutation frequency on M_1P basis =

$$\frac{\text{Sum of all the individual viable mutants in M2 generation} * 100}{\text{Total number of plants}}$$

Correlation- coefficient analysis was performed using IBM SPSS software 20.20 to estimate the relationship between M_1 estimates (lethality and injury) and mutation frequencies in M_2 generation.

RESULTS AND DISCUSSION

Effects of two mutagens *viz.,* ^{60}Co gamma rays, and ^{60}Co gamma rays+ EMS were studied individually and in combination on plant morphology of blackgram CO 6 variety. The deviation from general plant morphology includes a reduction in seed germination, survival rate (lethality), reduction in height of both seedlings and plant (injury), pollen, seed fertility percentage (fertility) which was found to contribute mutagenic efficiency. The mutagenic effect in M_1 generation was correlated with a mutation frequency of the M_2 population as reported by Prasath *et al*. (2019) in cluster bean.

A noteworthy factor used to estimate the mutagenic sensitivity is seed germination by which active metabolism is renewed after a certain period of dormancy (Shah *et al*., 2008). The response of the variety CO 6 to ^{60}Co gamma rays and combination treatment (^{60}Co gamma rays + EMS) for germination percentage revealed that all the treatments exhibited a dose-dependent negative linear relationship between dose and germination percentage (**Table 1**). The mean germination percentage ranged from 42.50 (400 Gy) to 83.72 (200 Gy) for ^{60}Co gamma rays whereas, it is varied from 36.71 (400 Gy + 30 mM) to 54.20 (200 Gy+20 mM) for combination treatments. Overall, ^{60}Co gamma rays treatments exhibited a low per cent of reduction (200 Gy:11.86 to 400 Gy:45.83) in comparison with combination treatments (^{60}Co gamma rays +EMS) of all doses (200 Gy+20 mM:36.88 to 400 Gy + 30 mM: 50.35).

All the mutagenic treatments showed a reduction in survival rate compared with control (**Table 1**). The survival percentage over control for gamma rays ranged from 52.08 (400 Gy) to 87.32 (200 Gy). Per cent reduction on survival rate was prominent in 400 Gy of gamma rays (47.92 %). In parallel, the reduction was found to be increasing at a higher dosage in ^{60}Co gamma rays + EMS combination with maximum reduction at 400 Gy + 30 mM (57.18 %). The mean values of survival percentage over control varied from 42.82 (400 Gy + 30 mM) to 60.80 (200 Gy+ 20 mM). The decrease in plant survival in the treated population may be associated with various factors such as cytogenetic damage and physiological disturbances and disturbances in balance between inhibitors of growth regulators and promoters (Meherchandani, 1975). The decline in survival percentage during later stage may be due to the inhibition of auxin synthesis (Skoog, 1935).

The data on the reduction in plant height on the 30th day due to the different doses of mutagen treatments are furnished in **Table 1**. In ^{60}Co gamma ray treatments, the mean plant height ranged between 15.16 cm (400 Gy) to 16.49 cm (200 Gy). The plant height reduction was maximum at 400 Gy (15.78 cm) and minimum at 200 Gy (8.39 cm). In case of combination (^{60}Co gamma ray +EMS), the mean plant height ranged from 15.94 cm (200 + 30 mM) to 12.98 cm (400 + 30 mM) and it showed a declining trend with increasing doses. The per cent reduction in plant height ranged from 11.50 (200 Gy + 20 mM) to 27.89 (400 Gy + 30 mM). According to Cherry and Lessman (1967), the reduction in plant growth may be due to decreased amylase activity, slow rate of cell division and enhanced peroxidase activity. Reduced plant height may also be attributed due to impaired cell division and cell elongation process, mutilated activity of mitosis in meristematic tissues (Khalil *et al*., 1986), destruction in ascorbic acid, and auxin content (biochemical, and physiological disturbances) (Gunckel and Sparrow, 1954).

Due to the mutagenic effects, the reduction in pollen fertility was observed in all the treatments in relation to control (92.20 %) (**Table 1 and Plate 1**). In ^{60}Co gamma rays, the pollen fertility percentage ranged from 48.37 (400 Gy) to 85.60 (200 Gy). The per cent reduction in pollen fertility was found to be maximum at higher doses (400 Gy) of gamma rays (40.28 %). A similar trend was noticed in combination (^{60}Co gamma rays +EMS) with the highest reduction at 400 Gy+30 mM (47.42 %). The fertility level of pollen ranged from 72.68 % (200 Gy + 20 mM) to 39.25 % (400 Gy + 30 mM) due to the combined mutagenic effect of ^{60}Co gamma rays +EMS.

The reduction in pollen fertility was comparatively higher in combination (^{60}Co gamma rays +EMS) than ^{60}Co gamma rays alone. The reduction in pollen fertility may be ascribed to upset in genetic equilibrium (cryptic deletions), point mutations (specific gene mutations) and cytoplasmic factors or production of non-viable pollens due to improper disjunction of the chromosome at anaphase (Khan and Wani, 2006). A dose-dependent reduction in chiasma intensity per cell and bivalents were documented by Gulfishan (2013) in Chillies, which led to sterility in pollen grains. Sharma and Reinbergs (1972) also revealed that the synergistic effects of combination of ^{60}Co gamma rays+ EMS enhanced seed sterility in barley.

The mean values of plant height at maturity varied from 47.45 cm (400 Gy) to 56.72 cm (200 Gy) due to the effect of ^{60}Co gamma rays. A higher per cent reduction in ^{60}Co gamma ray was noticed at 400 Gy (22.85 %) (**Table 1**). The combination (^{60}Co gamma rays +EMS) treatment induced more variation on plant height than gamma rays. In combination, the mean value of plant height ranged from 34.22 cm (400 Gy +30 mM) to 46.82 cm (200 Gy +20 mM). The maximum reduction was noticed at 400 Gy +30 mM of combination (^{60}Co gamma rays +EMS) with a 44.36 % reduction than ^{60}Co gamma ray (400 Gy: 22.85%) alone. All mutagenic combination treatments showed more reduction in plant height than ^{60}Co gamma rays treatment alone.

The reduction in plant height upon combined treatment was also reported by Bhosale *et al.* (2013) in blackgram. Irradiation may result in temporary arresting of cell division which may resume later depending on the intensity of the radiation. High radiation may destroy meristematic cells and inhibits cell division (Yadav, 2010). Elimination of damaged zones by inhibition of cell division at an earlier stage and replacement of injured meristematic cells may result in the reduction of plant height at maturity (Louis

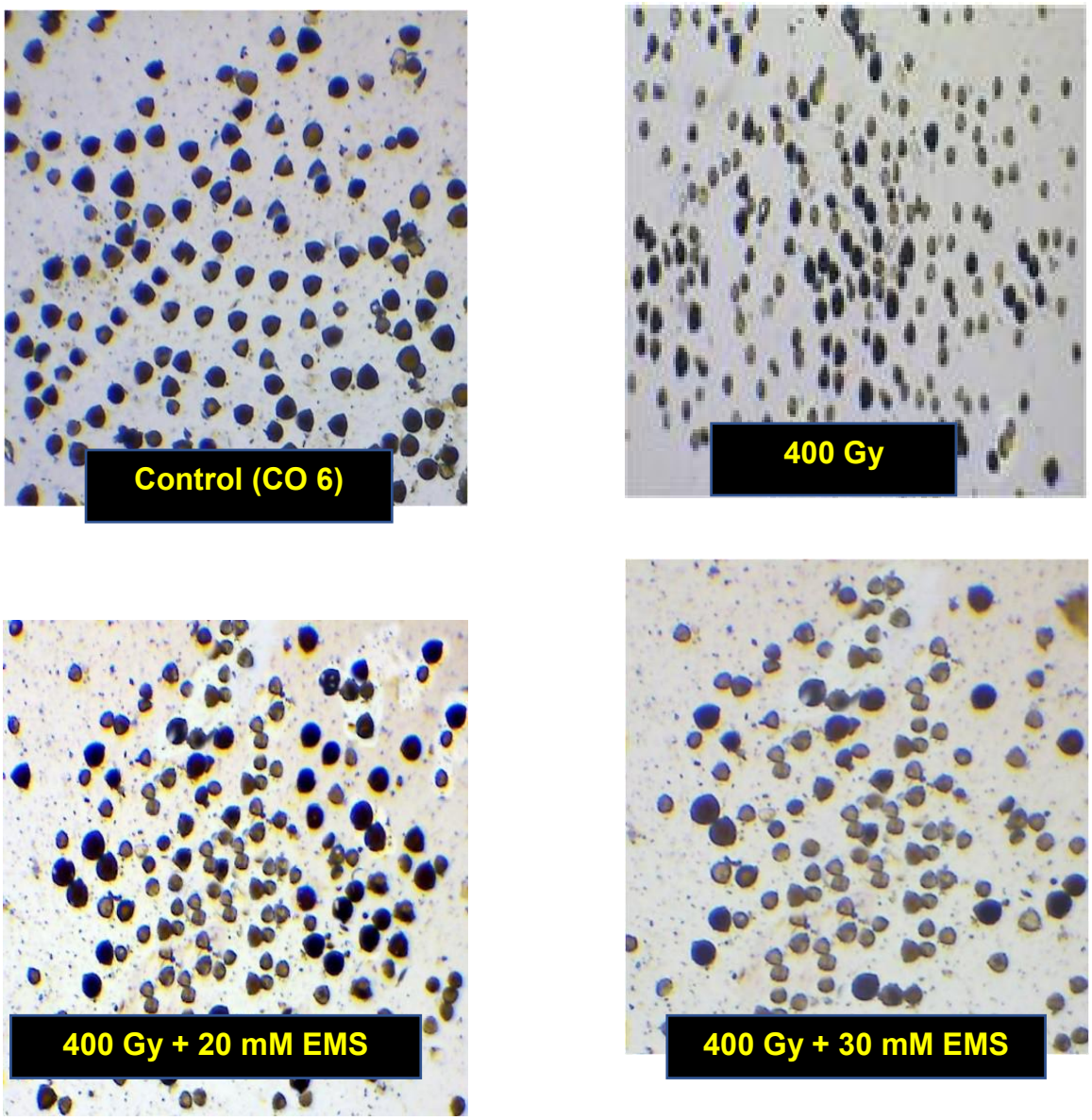

Plate 1. Effect of ^{60}Co gamma rays and ^{60}Co gamma rays + EMS at higher doses on pollen fertility in blackgram variety CO6

https://doi.org/10.37992/2021.1202.060

Table 1. Effect of mutagens on various growth factors in M_1 generation

Traits	Parameters		^{60}Co Gamma rays (Gy)			Combination [^{60}Co gamma rays (Gy) + EMS (mM)]					
		Control	200	300	400	200 + 20	200 + 30	300 + 20	300 + 30	400 + 20	400 + 30
Germination (%)	Mean	93.40 ±0.21	83.72±2.46	52.10±4.38	42.50±4.93	54.20 ±1.29	43.62 ±2.57	41.15 ±2.91	40.20 ±3.07	38.50 ±2.14	36.71 ±2.28
	Transformed mean	75.11	66.2	46.2	40.69	47.41	41.33	39.9	39.35	38.35	37.29
	Per cent over control	100	88.14	61.51	54.17	63.12	55.03	53.12	52.39	51.06	49.65
	Per cent reduction	-	11.86	38.49	45.83	36.88	44.97	46.88	47.61	48.94	50.35
Plant survival at 30th day	Mean	90.71 ±0.12	79.52±1.74	45.37±3.29	37.28±2.61	48.14 ±2.03	38.23 ±1.91	36.86 ±2.55	30.14 ±3.48	27.71 ±2.94	26.43 ±3.10
	Transformed mean	72.25	63.09	42.34	37.63	43.93	38.19	37.38	33.3	31.76	30.94
	Per cent over control	100	87.32	58.6	52.08	60.8	52.86	51.74	46.09	43.96	42.82
	Per cent reduction	-	12.68	41.4	47.92	39.2	47.14	48.26	53.91	56.04	57.18
Plant height at 30th day	Mean	18.00 ±0.19	16.49±1.76	15.72±1.32	15.16±2.95	15.93 ±2.39	15.94 ±2.13	15.80 ±1.98	15.20 ±2.14	14.40 ±3.61	12.98 ±2.97
	Per cent over control	100	91.61	87.33	84.22	88.5	88.56	87.78	84.44	80	72.11
	Per cent reduction	-	8.39	12.67	15.78	11.5	11.44	12.22	15.56	20	27.89
Plant height at maturity (cm)	Mean	61.50 ±0.46	56.72±1.38	49.96±1.24	47.45±3.61	46.82 ±2.15	44.64 ±1.38	44.89 ±3.19	40.23 ±2.32	38.91 ±1.60	34.22 ±2.54
	Per cent over control	100	92.23	81.24	77.15	76.13	72.59	72.99	65.41	63.27	55.64
	Per cent reduction	-	7.77	18.76	22.85	23.87	27.41	27.01	34.59	36.73	44.36
Pollen fertility (%)	Mean	92.20 ±0.47	85.60±1.63	57.41±2.15	48.37±3.42	72.68 ±2.69	59.72 ±3.16	56.30 ±2.19	51.50 ±3.03	43.00 ±2.85	39.25 ±4.51
	Transformed mean	73.78	67.7	49.26	44.07	58.49	50.6	48.62	45.86	40.98	38.79
	Per cent over control	100	91.75	66.77	59.72	79.27	68.59	65.9	62.16	55.54	52.58
	Per cent reduction	-	8.25	33.23	40.28	20.73	31.41	34.1	37.84	44.46	47.42
Seed fertility (%)	Mean	91.00 ±0.35	85.64±2.87	72.13±3.12	68.54±2.40	56.04 ±3.71	46.02 ±2.64	42.50 ±4.20	37.30 ±3.15	32.70 ±5.07	28.35 ±3.02
	Transformed mean	72.54	67.73	58.13	55.88	48.47	42.72	40.69	37.64	34.88	32.17
	Per cent over control	100	93.37	80.14	77.03	66.81	58.89	56.09	51.89	48.08	44.35
	Per cent reduction	-	6.63	19.86	22.97	33.19	41.11	43.91	48.11	51.92	55.65

and Kadambavanasundaram, 1973). The extreme dwarf plants were induced by combination treatment of ^{60}Co gamma + EMS which confirmed with the reports of Mishra *et al*. (2018) in rice bean.

A reduction in seed fertility percentage was observed in all mutagenic treatments compared to control (91.00 %). The per cent of seed fertility varied from 68.54 (400 Gy) to 85.64 per cent (200 Gy) due to the mutagenic effects of gamma rays. The maximum seed fertility reduction in ^{60}Co gamma ray was registered at 400 Gy (22.97 %). In ^{60}Co gamma rays + EMS, the seed set percentage ranged from 28.35 (400 Gy +30 mM) to 56.04 per cent (200 Gy+20 mM). The reduction in seed set percentage was found to be prominent in 400 Gy + 30 mM (55.65%) of ^{60}Co gamma rays + EMS (**Table 1**). The combination (^{60}Co gamma rays + EMS) treatment registered a maximum per cent reduction in seed fertility compared to all the treatments of ^{60}Co gamma rays. A dose-dependent increase in seed fertility reduction was observed.

Overall, the maximum reduction was noticed at a higher dose of ^{60}Co gamma rays (400 Gy) and a combination of ^{60}Co gamma rays + EMS (400 Gy +30 mM EMS) compared to other mutagenic treatments. Among the mutagens, a combination of ^{60}Co gamma ray + EMS exhibited maximum effect with a high per cent reduction in all the traits under study. This was in agreement with earlier reports of Veni *et al*. (2017) in blackgram and Prasath *et al*. (2019) in cluster bean. All the parameters recorded in this study were found to be decreasing with increased doses of mutagens and the sensitivity of the selected genotype to ^{60}Co gamma rays + EMS is higher than ^{60}Co gamma rays.

Viable mutation frequency was found to be directly proportional to the doses of mutagen in ^{60}Co gamma rays and ^{60}Co gamma rays + EMS (**Table 2**). Among the nine treatments, high mutation frequencies were observed in 400 Gy of ^{60}Co gamma rays and 400 Gy ^{60}Co gamma rays + 30 mM EMS, whereas the combination of ^{60}Co gamma rays + EMS recorded higher mutagenic frequency than the individual treatments. As a result, the current investigation has successfully demonstrated in establishing a mutagenized population with wider genetic diversity for the traits namely plant height, leaf shape, size, duration, length of the pod, and yield. The identified putative mutants will be advanced to next generation for further evaluation and the stabilized mutants can be used in a cross-breeding programme to transfer the trait of interest.

Table 2. Viable mutation frequency in M_2 generation

Mutagens	Number of M_1 families		Number of M_2 plants		Mutation frequency	
(Dose / Conc.)	Scored	Segregated	Scored	Segregated	Per 100 M_1 plants	Per 100 M_2 plants
^{60}Co gamma rays (Gy)						
Control	80	-	1300	-	-	-
200	115	7	1710	13	6.09	0.76
300	115	10	1629	18	8.70	1.10
400	115	13	1574	25	11.30	1.59
^{60}Co gamma rays (Gy) + EMS (mM)						
Control	80	-	1300	-	-	-
200+20	115	12	1592	27	10.43	1.70
200+30	115	15	1523	32	13.04	2.10
300+20	115	17	1442	32	14.78	2.22
300+30	115	21	1426	34	18.26	2.38
400+20	115	26	1372	45	22.61	3.28
400+30	115	32	1336	51	27.83	3.82

Table 3. Correlation coefficients of mutagenic sensitivity factors in M_1 generation with M_2 viable mutation frequency

S.No.	Parameters	Mutagenic sensitivity factors	Mutation frequency (%)
1.	Lethality	Germination (%)	0.70*
2.		Plant survival (%)	0.78*
3.	Injury	Plant height at 30 DAS	0.89**
4.		Plant height at 30 DAS	0.96**

* Significance at 5% level; ** Significance at 1 % level

A closer examination of the data (**Table 2**) revealed a linear dependency between the viable mutation frequency in the M_2 generation and biological damage caused by mutagens in M_1 generation. Akin findings were reported by Selvam *et al.* (2010) and Singh and Mohapatra, (2004). Correlation coefficients were further used to investigate the association between average estimates of M_1 parameters namely lethality, injury, and mutation frequency in M_2 (**Table 3**).

A significant positive correlation was observed between the viable mutation frequency and estimates in M_1 generation (**Table 3**). As a result, lethality and injury were presumed to be potential markers of M_2 generation mediated mutation frequency. Hence, higher doses of ^{60}Co gamma rays (300 & 400 Gy) and ^{60}Co gamma rays +EMS (300 Gy+ 30; 400 Gy+ 20 and 400 Gy+ 30 mM) proved to be efficient in increasing the mutation frequency towards desirable directions. These results were largely in line with previous findings (Singh and Rao, 2007; Singh and Mohapatra, 2004).

From the results of the present study, it is concluded that there was a significant variation between the mutant and the control population in M_1 generation due to combination treatments of ^{60}Co gamma rays +EMS and ^{60}Co gamma rays. Both the growth characteristics and biological responses decreased with an increasing dose for all the treatments. The linear dependency was observed between the viable mutation frequency in M_2 generation and induced biological damage in M_1 generation. Hence, higher doses of ^{60}Co gamma rays and ^{60}Co gamma rays + EMS proved to be efficient in increasing the mutation frequency towards desirable directions. Comparing to gamma rays treatment, the traits under study, showed a marked reduction in combination treatments (^{60}Co gamma rays + EMS) which could be useful in inducing a wide range of desirable mutations in crops.

ACKNOWLEDGMENT

The authors would like to acknowledge GOI-BRNS, BARC, Mumbai for funding the research work.

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