Comparative Mutagenic Analysis of Gamma Rays, EMS and Their Combination Treatments in Black Gram (*Vigna mungo* (L.) Hepper)

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ABSTRACT

Present investigation was carried out to study the individual and combined effects of gamma rays and ethyl methanesulphonate (EMS) on various biological parameters such as seed germination, plant survival, pollen fertility and seedling height in M_1 generation of black gram (*Vigna mungo* (L.) Hepper) varieties T-9 and Pant U-30. A dose dependent decrease in these parameters, except plant survival at maturity was observed in both varieties. Gamma ray treatments induced greater reduction in seed germination, pollen fertility and seedling height than EMS treatments. However, combination treatments of gamma rays and EMS caused maximum reduction as compared to their individual treatments vis-à-vis aforesaid parameters. Morphological investigation of M_1 seedlings revealed various types of anomalies in cotyledonary leaves. The frequency of Type-II anomalies was the highest followed by Types-I and III in both varieties. All these abnormal seedlings, except Type-I were normal plants in flowering and set seeds. Combined treatments induced the highest frequency of such abnormalities in both varieties.

Keywords: Mutagenesis, gamma rays, EMS, radio-sensitivity, chemo-sensitivity, black gram

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INTRODUCTION

Grain legumes, commonly known as pulses, occupy a pivotal position in meeting the protein needs of masses in developing countries like India. They have proved to be the nutrient dense food stuffs especially the source of vegetable proteins. With population growing at rapid rate, the stagnation in pulse production has created a severe decline in its per capita availability- a matter of serious concern in a country where pulses are the major source of proteins for most of the people. The availability of pulses has declined because their production could not keep pace with the population growth. It is a fact that Indians today are consuming far less proteins than they used to in past. Per capita pulses consumption over the years has significantly come down from 60.7 g/day in 1951 to 43.3 g/day in 2013 (Annual Report, 2016−2017). Solution to the problem of declining per capita availability has to come from rapid improvement in the indigenous production levels. While efforts have been geared up to bring additional area under the cultivation of pulses, however more important is to increase the production by exploiting the yield potential of existing varieties through genetic manipulation.

Leguminous crops add more nitrogen to our soils than the total nitrogen fertilizer being produced in the country (Rehman, 2000). Since black gram belongs to the leguminous group of plants, it too shares this advantage. It possesses deep penetrating root system which enables it to utilize the limited resource of moisture content more efficiently than many other crops including cereals, and contribute substantially to the loosening of the soil for better aeration (Singh *et al.*, 2011; Solanki *et al.*, 2011). Because of this property, farmers choose to grow black gram under highly diversified conditions. Moreover, it differs from other pulse crops in its peculiarity of attaining mucilaginous pasty character when soaked in water. In south India, black gram is used in the preparation of variety of dishes like vada, idli, dosa, halwa, imarti in combination with other food grains.

Black gram crop prefers water retentive stiff or heavy soil and does well on both black cotton and brown alluvium soils with pH range of 4.7–7.5 (Rehman, 2000). In India, black gram was grown over an area of 3.15 million hectares with the production of 1.97 million tonnes in 2012–2013. The average yield of 625 kg/ha was low as compared to other pulse crops viz., fieldpea-1099 kg/ha, chickpea-1036 kg/ha, lentil-797 kg/ha, pigeonpea-776 kg/ha (Annual Report, 2016−2017) and was not sufficient to meet the growing demand of the people. In order to break the yield plateau in black gram, efforts are needed to develop high yielding varieties with appropriate growth habit. The possibility offered by mutagenic agents to induce new genetic variation, is therefore of extreme interest.

Mutations are the ultimate source of genetic variation (Stebins Jr, 1950). For plant breeding purposes, mutations are of interest in two different ways. Firstly, they provide us with new starting material (building blocks) for the production of new cultivars and give tools for identifying new genes, and secondly they are useful in studying the nature of genes and their ways of controlling biochemical pathways (Konzak, 1984; Konzak *et al.*, 1984; Rick, 1986; Old and Primrose, 1989; Micke *et al.*, 1990). Although the mechanism of mutagenesis in higher plants is still not fully understood, yet it is clear by now that errors in replication, recombination, repair and damage in DNA are involved. Other phenomenon like transposition effect caused by the transfer of DNA segment to another position may result in modification or inactivation of gene action, thus induces heritable changes or mutations (Van Harten, 1998).

In recent years, a lot of work has been undertaken on induced mutagenesis through physical and chemical mutagens in various crop plants (Ciftci *et al.*, 2006; Ali and Sheikh, 2007; Girija *et al.*, 2013; Ariraman *et al.*, 2015; Wani, 2017a; Gupta *et al.*, 2018; Julia *et al.*, 2018; Laskar *et al.*, 2018). It has been clearly shown in number of plant species that the effect induced varies with varying mutagens and doses. Thus, selecting a mutagen and its optimum dose for a genotype in any plant species is an important step in mutation breeding research (Konzak *et al.*, 1965; Solanki and Waldia, 1997; Van Harten, 1998). Understanding the relevance of the subject, an attempt has been made to study the biological damage and cotyledonary abnormalities in M_1 generation of black gram using single and combined treatments of gamma rays and EMS.

MATERIALS AND METHODS

Two varieties of black gram (*Vigna mungo* (L.) Hepper) viz., T-9 and Pant U-30 were used in the present study. Seeds of both the varieties were obtained from G.B. Pant University of Agriculture and Technology, Pantnagar, Uttaranchal, India. Both the varieties are well adapted to agro-climatic conditions of Uttar Pradesh, India including the study site. Dry seeds of each variety, with moisture content of 12%, were irradiated with 100, 200, 300 and 400 Gy doses of gamma rays at a dose rate of 0.16 Gy/second from a radioisotope ^{60}CO (Cobalt-60) source at National Botanical Research Institute (NBRI), Lucknow, Uttar Pradesh, India. The percentage of moisture content was determined following international seed testing association (ISTA, 1985) guideline which is based on the difference between fresh and dry weight of the seeds.

For chemical treatments, healthy seeds of uniform size of each variety were presoaked for 9 hours in distilled water and treated with 0.1, 0.2, 0.3 and 0.4% of ethyl methanesulphonate

(EMS-a monofunctional alkylating agent, manufactured by Sissco Research Laboratories Pvt. Ltd., Mumbai, India) for 6 hours with intermittent shaking at room temperature of 25 ± 1°C. Lethal dose-50 (LD_{50}) was determined to find out the sensitivity of genotypes towards the mutagens/ mutagenic treatments. The solution of EMS was prepared in phosphate buffer of pH 7. After treatment, the seeds were thoroughly washed in running tap water to remove the residual mutagen from seed surface. For combination treatments, dry seeds of each variety were firstly irradiated with gamma rays at 200 and 300 Gy doses and then treated with 0.2 and 0.3% EMS (i.e. 200 Gy + 0.2% EMS, 300 Gy + 0.2% EMS, 200 Gy + 0.3% EMS and 300 Gy + 0.3% EMS). 350 seeds were used for each treatment and controls.

Three replications of 100-seeds each were sown for every treatment and control in each variety in a randomized complete block design (RCBD) at the Agriculture Farm, Aligarh Muslim University, Aligarh, India. The spacing was maintained at 30 cm (seed to seed in a row) and 60 cm (between the rows) in the field. The experiment was conducted during zaid (summer) season of 2008. Recommended agronomic practices were employed for preparation of field, sowing and subsequent management of the population of black gram.

The percentage of seed germination was calculated on the basis of total number of seeds (300) sown in the field and the number of seeds germinated afterwards. The remaining lot of 50 seeds was sown on moist cotton in petriplates and kept in BOD incubator at 27 ± 1°C temperature. After 10 days of sowing the seeds in petriplates, growth observations were recorded on shoot + root lengths of seedlings. Seedling injury was measured in terms of reduction in seedling height with respect to controls.

Pollen fertility was determined from 30 randomly selected plants (10 from each replicate) from each treatment and control at the time of flowering. Ten randomly selected flowers from each replicate/treatment were used for the studies of pollen fertility. Pollen grains were stained with 1% acetocarmine solution and observed under olympus compound microscope. Pollen grains which took stain and had regular outline were considered as fertile, while the shrunken and unstained ones as sterile. The surviving plants in different treatments and control were counted at the time of maturity and the survival percentage was computed in relation to percentage of seeds germination in the field.

Following formula was used to calculate the percentage inhibition for various biological parameters:

$$\text{Percentage inhibition} = \frac{\text{Control} - \text{Treated}}{\text{Control}} \times 100$$

The frequency of cotyledonary abnormalities in various treatments was calculated by the following formula:

$$\text{Cotyledonary abnormalities (\%)} = \frac{\text{No. of seedlings showing cot. Abnormalities}}{\text{Total number of } M_1 \text{ seedlings}} \times 100$$

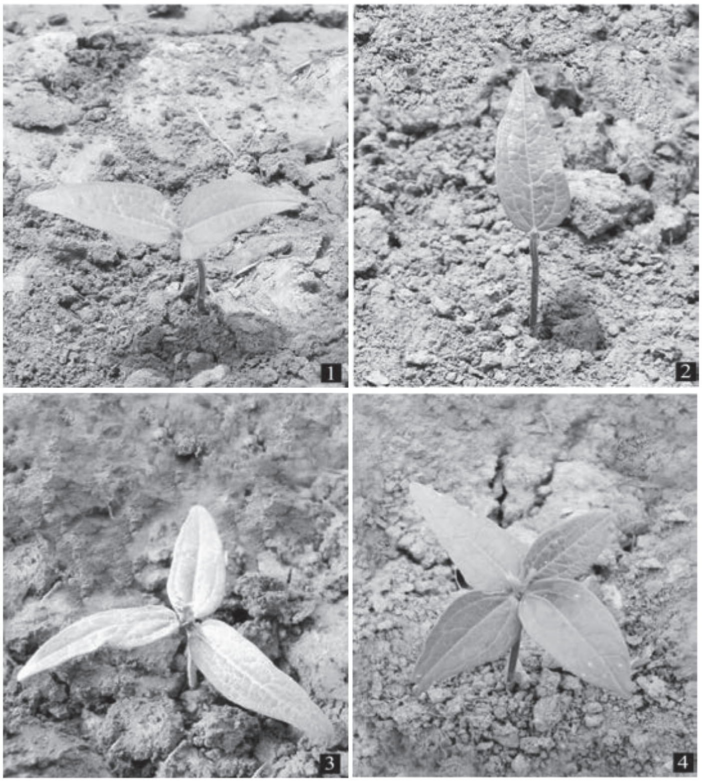

Figure 1 Seedlings of control and treated population of black gram with varied number cotyledonary leaves. (1) Seedling with normal pair of cotyledonary leaves (control) (2) Seedling with one cotyledonary leaf (3) Seedling with an extra cotyledonary leaf (4) Seedling with an extra pair of cotyledonary leaves

RESULTS AND DISCUSSION

Data recorded on seed germination is presented in Table 1 and Figure 2. A gradual decrease in seed germination was observed with increasing doses of the mutagens in both the varieties. The varieties however, differed in the extent of reduction in seed germination. Seed germination was affected more adversely in the variety Pant U-30 as compared to variety T-9. Combined (gamma rays + EMS) treatments were found to cause maximum reduction in seed germination followed by individual treatments of gamma rays and EMS. The percentage inhibition was 5.26 and 36.84 with 200Gy + 0.2% EMS and 300Gy + 0.3% EMS, respectively in the variety Pant U-30, whereas it was 4.17 and 35.42 with these treatments in the variety T-9. Germination started the fourth day after sowing in control of both the varieties. However, it was delayed by more than two days in the lots treated with higher doses of mutagens. The delay in seed germination was found to be more pronounced in combined treatments.

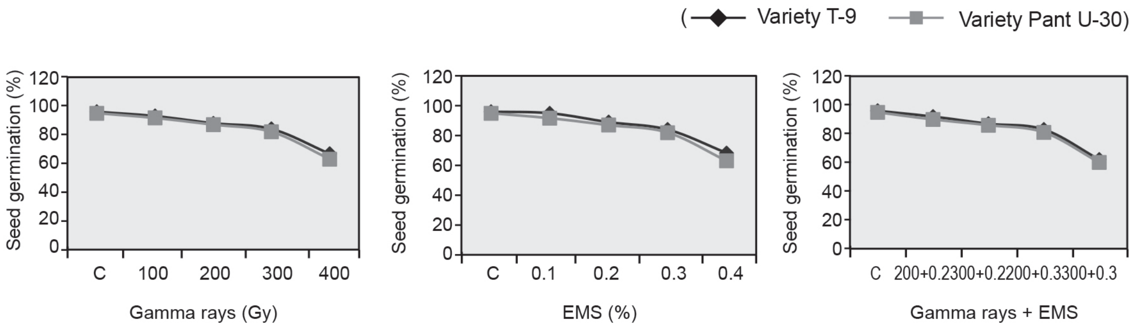

Figure 2 Effects of mutagens on seed germination (%) in M_1 generation of black gram

Table 1 Effects of gamma rays, EMS and their combination on various biological parameters in M_1 generation of black gram

Treatment	Variety T-9					Variety Pant U- 30				
	Seed germination (%)	% age inhibition	Plant survival (%)	Pollen fertility (%)	% age reduction	Seed germination (%)	% age inhibition	Plant survival (%)	Pollen fertility (%)	% age reduction
Control	96.00	-	92.01	98.25	-	95.00	-	91.23	97.20	-
Gamma rays										
100 Gy	93.00	3.12	89.60	91.65	6.72	91.67	3.50	88.01	90.00	7.41
200 Gy	88.00	8.33	82.19	85.40	13.08	87.00	8.42	80.46	83.12	14.48
300 Gy	84.00	12.50	83.33	80.22	18.35	82.00	13.68	81.30	76.19	21.61
400 Gy	66.67	30.55	62.50	74.12	24.56	63.00	33.68	60.53	70.20	27.78
EMS										
0.1%	95.00	1.04	89.82	92.00	6.36	93.00	2.10	87.50	89.23	8.20
0.2%	89.00	7.29	82.40	85.45	13.03	88.00	7.37	77.65	84.00	13.58
0.3%	84.00	12.50	83.33	80.65	17.91	84.00	11.58	79.36	77.01	20.77
0.4%	68.33	28.82	62.44	74.35	24.32	65.00	31.58	61.54	71.05	26.90
Gamma rays+EMS										
200 Gy+0.2%	92.00	4.17	84.06	85.00	13.49	90.00	5.26	81.48	84.20	13.37
300 Gy+0.2%	87.00	9.37	86.21	79.05	19.54	86.00	9.47	79.46	77.94	19.81
200 Gy+0.3%	83.00	13.54	82.33	75.87	22.78	81.00	14.74	80.25	71.17	26.78
300 Gy+0.3%	62.00	35.42	59.14	70.15	28.60	60.00	36.84	58.33	69.25	28.75

Percentage of plant survival decreased gradually in all mutagenic treatments (Table 1 and Figure 3). However, it was dose independent. The highest plant survival was observed in the control population of both the varieties. The studies of pollen fertility in mutagen treated population forms a reliable index in assessing any internal change in plants as well as in determining the efficacy of a mutagen.

Although 2–3% of pollen sterility was observed in control plants, yet the reduction in pollen fertility in mutagen treated population was quite high and dose dependent in both the varieties (Table 1 and Figure 4). The highest percentage of reduction was recorded in combined treatments of gamma rays + EMS followed by individual treatments of gamma rays and EMS. The fertility was lowest (70.15 and 69.25 percent) with 300Gy + 0.3% EMS in varieties T-9 and Pant U-30, respectively. Greater reduction in pollen fertility was observed in variety Pant U-30 than the variety T-9.

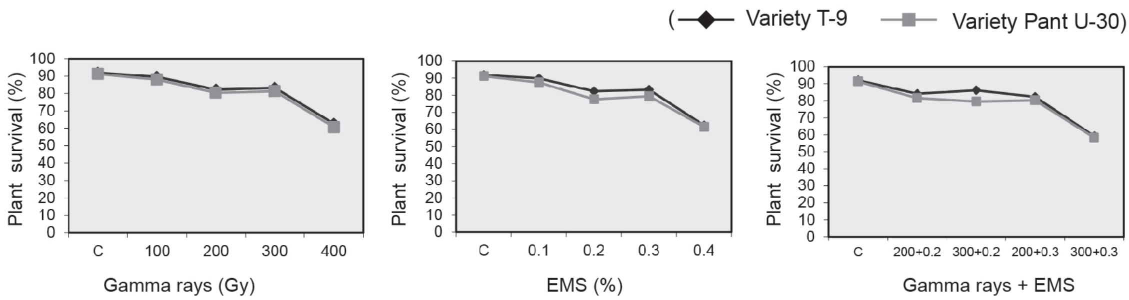

Figure 3 Effects of mutagens on plant survival (%) in M_1 generation of black gram

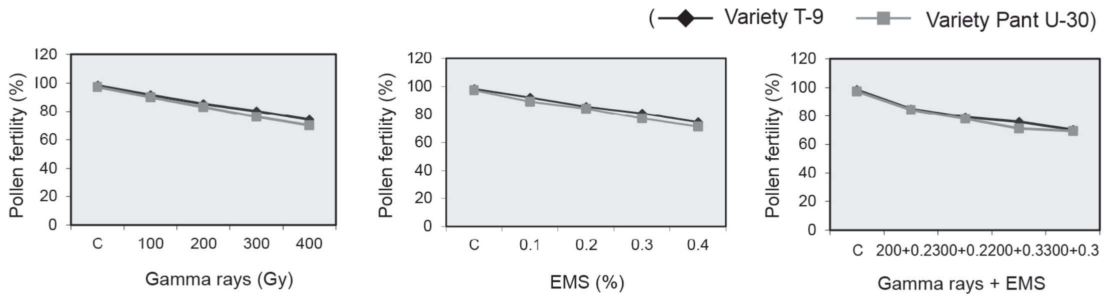

Figure 4 Effects of mutagens on pollen fertility (%) in M_1 generation of black gram

Data on seedling height (shoot + root) presented in Table 2 and Figure 5, show that all the mutagenic treatments caused reduction in seedling height in both the varieties. The reduction was more pronounced in combination treatments as compared to individual treatments of gamma rays and EMS. Percentage injury in seedling height increased with increasing doses of mutagen in both the varieties. Variety T-9 was relatively more sensitive with respect to seedling injury. In var. T-9, the seedling injury ranged from 10.41 to 36.96% with the treatments of gamma rays and 8.69 to 34.06% with EMS treatments. For combination treatments, the seedling injury ranged from 11.59 to 41.75%.

Table 2 Effects of gamma rays, EMS and their combination on seedling height (cm) in M_1 generation of black gram

Treatment	Variety T-9			Variety Pant U- 30		
	Range	Mean	% age injury	Range	Mean	% age injury
Control	8.00–27.00	16.91	-	7.00–26.00	17.25	-
Gamma rays						
100 Gy	6.30–19.00	15.15	10.41	5.00–25.50	15.55	9.85
200 Gy	6.70–24.20	14.64	13.42	5.00–22.20	15.00	13.04
300 Gy	4.50–18.90	11.78	30.34	3.90–15.00	12.78	25.91
400 Gy	1.20–14.00	10.66	36.96	3.20–13.20	10.25	40.58
EMS						
0.1%	6.40–24.10	15.44	8.69	5.50–25.00	16.00	7.25
0.2%	5.75–24.30	14.70	13.07	4.50–23.20	15.20	11.88
0.3%	6.50–17.50	13.28	21.47	2.20–15.50	14.35	16.81
0.4%	4.50–14.90	11.15	34.06	3.20–13.50	12.50	27.54
Gamma rays+EMS						
200 Gy+0.2%	6.00–19.00	14.95	11.59	3.80–16.10	15.49	10.20
300 Gy+0.2%	4.50–18.70	14.05	16.91	3.80–15.50	14.85	13.91
200 Gy+0.3%	1.50–19.50	11.25	33.47	2.50–12.50	11.65	32.46
300 Gy+0.3%	1.20–10.50	9.85	41.75	2.00 – 9.20	10.15	41.16

The frequency of different cotyledonary abnormalities recorded at seedling stage in the field is given in Table 3, Figure 6 and Figure 1. The characteristics of cotyledonary anomalies are given below:

Type-I : Seedlings with one cotyledonary leaf instead of a pair in normal case.
Type-II : Seedlings with an extra cotyledonary leaf.
Type-III : Seedlings with an extra pair of cotyledonary leaves.

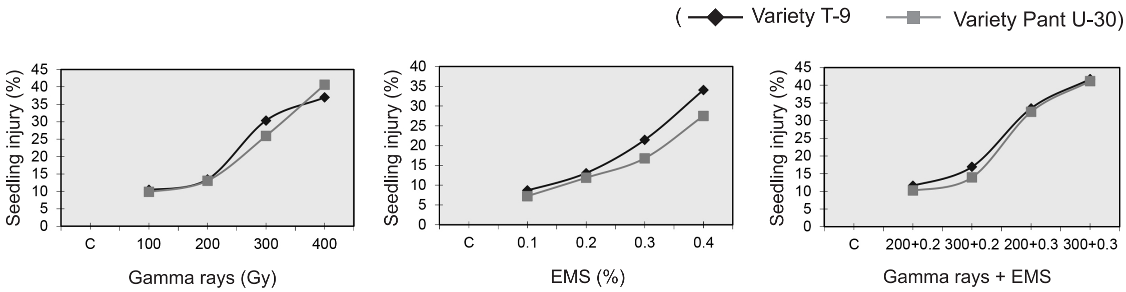

Figure 5 Effects of mutagens on seedling injury (%) in M_1 generation of black gram

Variations in number of cotyledonary leaves were recorded with different mutagenic treatments. The frequency of Type-II abnormalities was the highest followed by Type-I and III in both the varieties. All these abnormal seedlings except Type-I were normal plants in flowering and set pods and seeds. Combined treatments of gamma rays and EMS induced the highest frequency of such anomalies in both varieties.

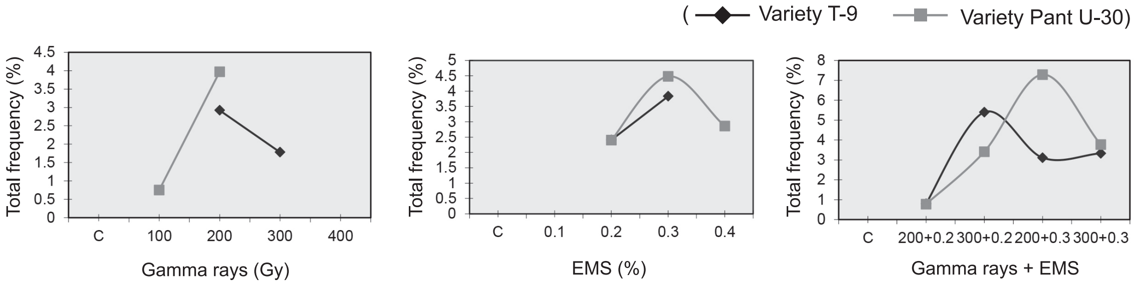

Figure 6 Frequency of cotyledonary abnormalities (%) in M_1 generation of black gram

Gamma rays are the most energetic forms of electromagnetic radiations which can be useful for the alteration of physiological characters in plant species (Chaudhuri, 2002; Kovacs and Keresztes, 2002; Kiong *et al.*, 2008). These radicals can damage or change the important components of plant cells. They have been reported to affect differentially the morphology, anatomy, biochemistry and physiology of plants depending upon the radiation dose (Ashraf *et al.*, 2003). EMS-an alkylating agent produces random mutations in genetic material by nucleotide substitution, particularly by guanine alkylation and is reported to be the most effective and powerful mutagen in producing point mutations (Hajara, 1979; Okagaki *et al.*, 1991).

The studies of biological damage in terms of seed germination, survival at maturity, pollen fertility, seedling growth depression and cotyledonary abnormalities in M_1 generation are generally used to evaluate the mutagenic sensitivity of biological system. It was observed during mutagenic studies that gamma rays and EMS employed alone as well as in combination brought about reduction in seed germination, survival at maturity, pollen fertility and seedling height. Such reductions, with an exception of plant survival, were found to be dose dependent. Earlier studies of Mahna *et al.* (1989) and Goyal and Khan (2010) in *Vigna mungo*, Gaikwad and Kothekar (2004) and Wani (2017b) in *Lens culinaris*, Khan and Wani (2005) in *Vigna radiata,* Toker and Cagirgan (2004) and Barshile *et al.* (2006) and Wani *et al.* (2012) in *Cicer arietinum*, Ariramana *et al.* (2014) in *Cajanus cajan* and Vedna *et al.* (2016) in *Sesamum indicum* have shown linear relationship between the mutagen dose and the parameters studied above.

Seed germination decreased with increasing doses of the mutagens. The reduction was slightly more in gamma ray treatments as compared to EMS treatments. Gamma irradiation is known to cause disruption and disorganization of the tunica layer, thus inhibiting the seed germination (Chauhan and Singh, 1975). The most striking effect is the impairment of cell division in the meristematic zone during the germination process of irradiated seeds (Cherry and Hageman, 1961). A decrease in respiratory quotient in irradiated seeds may also inhibit the germination (Woodstock and Justice, 1967). Reduced germination due to mutagenic treatments, in the present study, may be the result of delay or inhibition of metabolic activation necessary for seed germination. Reduction in germination was more drastic in combination treatments as compared to single treatments, indicating a synergistic effect by enhanced toxicity. Delayed germination by more than two days in the lots treated with high doses of mutagens may be attributed to inhibition of mitotic proliferation in root and shoot meristems.

Table 3 Frequency of cotyledonary abnormalities in M_1 generation of black gram

Treatment	Variety T-9			Variety Pant U- 30		
	Type-I	Type-II	Type-III	Type-I	Type-II	Type-III
Control	-	-	-	-	-	-
Gamma rays						
100 Gy	-	-	-	-	0.75	-
200 Gy	1.67	1.25	-	2.38	1.59	-
300 Gy	1.78	-	-	-	-	-
400 Gy	-	-	-	-	-	-
EMS						
0.1%	-	-	-	-	-	-
0.2%	-	2.41	-	-	2.40	-
0.3%	2.13	1.70	-	2.00	2.48	-
0.4%	-	-	-	2.86	-	-
Gamma rays+EMS						
200 Gy+0.2%	0.80	-	-	-	0.78	-
300 Gy+0.2%	2.56	2.41	0.43	2.13	-	1.28
200 Gy+0.3%	-	3.11	-	2.19	2.63	1.75
300 Gy+0.3%	-	2.00	1.33	-	1.26	2.51

The survival of plants decreased appreciably in the mutagenic treatments in both the varieties, however there was no direct relationship between the dose and survival of plants. These results are contrary to the earlier findings of Lal *et al.* (2009) who reported a linear relationship between plant survival and mutagen doses in urdbean. The reduction in survival percentage in the treated population could be due to disturbed physiological processes or chromosomal damage leading to mitotic arrest.

The magnitude of pollen sterility, in the present study, increased linearly with increasing doses of the mutagens. These findings are in agreement with the earlier reports in *Lens culinaris* (Wani *et al.*, 2004), *Cicer arietinum* (Barshile *et al.*, 2006), *Vigna radiata* (Khan and Wani, 2006) and *Vigna mungo* (Sharma *et al.*, 2006). In most of the cases, meiotic abnormalities are responsible for pollen sterility (Gaul, 1970; Sinha and Godward, 1972; Gottschalk and Klein, 1976; Dhamyanthi and Reddy, 2000; Kumar and Singh, 2003).

Decrease in seedling height with increasing mutagenic doses is likely due to chromosomal damage or inhibition of cell division. Reddy *et al.* (1992) observed that seedling height reduction may be due to chromosomal breakage during the mitotic divisions. The reduction of seedling height is attributed to auxin destruction, changes in ascorbic acid content and physiological injury in the seeds and seedlings (Usuf and Nair, 1974). Combination treatments showed greater injury to seedling height as compared to individual treatments of gamma rays and EMS. This is in conformity with the earlier report of Ignacimuthu and Babu (1988).

The seedlings of black gram normally emerge with two juvenile cotyledonary leaves oriented opposite to each other on the axis. The mutagens (gamma rays and EMS), in this study, induced variation in the number of cotyledonary leaves in both the varieties. The factors responsible for induction of such abnormalities are not well known. However, according to Devreux and Mugnozza (1964), the general disturbance in metabolic pathways due to irradiation could be the main reason for such abnormalities. The presence of single cotyledonary leaf in seedlings may be due to cytochemical disturbances or acute chromosomal aberrations leading to death of leaf primordia or embryonal cell responsible for leaf development. The formation of an extra cotyledonary leaf, on the other hand, indicates the formation and involvement of additional leaf primordia or the embryonal cell. Most of these abnormalities in cotyledonary leaves did not appear in M_2 population. Presumably, in the present study, the anomalies in cotyledonary leaves were induced due to mutagenic toxicity or other physiological disturbances as also suggested by Mahna *et al.* (1989) in black gram.

CONCLUSION

Estimation of induced biological damage helps in determining the sensitivity of a biological material as well as the potency of a mutagen. In the present investigation, seed germination was affected more adversely in the variety Pant U-30. Combined treatments of gamma rays and EMS caused maximum reduction in seed germination as compared to their individual treatments. Plant survival gradually decreased with all the mutagenic treatments in both the varieties. Even if 2–3% of pollen sterility was observed in control plants, still the reduction was quite high and dose dependent in treated population. Variety T-9 was relatively more sensitive with respect to seedling injury. Combined treatments of gamma rays and EMS induced highest frequency of cotyledonary abnormalities. Mutagenic treatment, causing least biological damage in M_1 generation, generally shows high degree of effectiveness and efficiency in inducing desirable mutations in subsequent generations. Though black gram seedlings are bi-foliate in nature, the increase in the number of cotyledonary leaves will increase the biomass production which could positively impact the overall growth of the plant.

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