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Effect of Chemical and Physical Mutagen in M1 Generation and Chlorophyll Mutations in Soybean (*Glycine max* L. Merrill.)

Digambar Ahire*[1] and Sanjay Auti[2]
[1]Department of Botany, New Arts, Commerce and Science College, Ahmednagar, India
[2]Department of Botany, H.PT. Arts and R.Y.K Science College, Nashik-422005, India

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Abstract: Soybean variety 'MACS 450' was subjected to combination treatments of gamma rays (50 Gy and 100 Gy) and EMS (0.2% and 0.4%), different presoaked hours (12hrs, 16hrs and 20hrs) with 0.8% EMS, and gamma rays (50 Gy) with sodium azide (0.003 % and 0.005%) with an objective to induce variability for quantitative traits and estimate frequency and spectrum of chlorophyll mutations. In M_1 generation effect of mutagenic treatments was assessed on the basis of parameters survival (%), plant height reduction and seed sterility against control. Overall compare to all combination treatments 100Gy +0.4% EMS exerted more injurious effects in M_1 in the form of reduction in height (36.4%) and pod /plant (35.3). Among combination treatments of gamma rays + EMS and EMS treatment to presoaked seeds, the highest frequency of chlorophyll mutations (2.91% and 4.50%) was observed in 100 Gy + 0.4 % EMS and 16hrs PSW + 0.8% EMS respectively on M_2 plant basis. The results indicated that both these treatments are most effective to induce a wide range of genetic variability in soybean.

Key words: EMS; Sodium azide; Gamma rays; M1 generation; Chlorophyll Mutations; Soybean

Introduction

Glycine max (L.) Merrill, is an annual leguminous species cultivated mainly for its seeds, that occupies a coveted place among the oilseed crops, being cultivated all over the world, is an economically important leguminous crop for oil, feed and food products. In India, soybean attributes above 10 percent to the domestic edible oil pool, and the country earns substantial foreign exchange through export of soy meal (Joshi, 2003). It has become a major oil seed crop of India, covering an area of about 6.0 million hectares with 5.5 million tones as annual production. Soybean ranks first among the major oilseed crops of the world and has now found a prominent place in India (Mahna, 2005). Though the area and production has increased, the productivity is less than half as compared to the world average. The major constraints for low productivity of soybean are its poor seed viability and non-availability of early maturing, photo-insensitive, high yielding cultivars with resistance to biotic and abiotic stresses (Bhatnagar and Karmakar, 1995). Secondly the present day soybean cultivars are derived from narrow genetic base. The genetic variability present in any crop is of vital importance in the formulation of effective breeding programme. Thus, the genetic variability generated by induced mutations can certainly help to recover the alleles for higher productivity and also for better plant type. The present studies were undertaken to induce mutations in the cultivar 'MACS 450' and estimate frequency and spectrum of chlorophyll mutations which is considered as a dependable index for evaluating the efficiency and effectiveness of the mutagens and their effective concentration in mutation breeding.

Materials and Methods

Dry seeds of soybean variety 'MACS 450' were exposed to gamma rays in gamma cell 200 with ^{60}Co source installed at BARC. Gamma rays treated (dose 50 Gy and 100 Gy) seeds were subjected to EMS (0.2 and 0.4%) for combination treatments of gamma rays and EMS (50Gy + 0.2% EMS, 50Gy + 0.4% EMS, 100Gy + 0.2% EMS and 100Gy + 0.4% EMS). In another experiment, seeds irradiated with gamma rays at 50Gy were treated with various concentrations of sodium azide (0.003 % and 0.005 % w/v NaN_3) for combination treatments of gamma rays and sodium azide (50Gy + 0.003 % NaN_3, 50Gy + 0.005 % NaN_3). Untreated soybean seeds were presoaked in distilled water for varied time duration viz, 12 hours, 16 hours and 20 hours. They were made DNA synthetically active as in presoaked seed treatments. The presoaked seeds were then treated with 0.8% (v/v) EMS solution. For each treatment of EMS and sodium azide, seeds were treated for 4hrs in dark at 24±2 °C. EMS solution was prepared in phosphate buffer (pH 7.0) and the sodium azide was prepared in phosphate buffer (pH 3.0) which is the most favorable pH for treatment with NaN_3 (Gichner *et al.*, 1994). The 0.8% EMS concentration was selected on the basis of (LD 50) lethal dose from pre experiments. After treatment and 4 hrs post soaking, the treated seeds were thoroughly washed in running tap water for 2 hours. The treated seeds along with the control were sown in the experimental field following randomized block design (RBD) with three replications at Department of Botany, University of Pune to raise the M_1 generation. The data on germination were recorded at three to twelve days after sowing. Observations were recorded on survival (%), plant height, pods/ plant, seeds/ plant and seeds/

***Corresponding Author:**
Prof. Digambar Ahire,
Department of Botany,
New Arts, Commerce and Science College,
Ahmednagar, India.

pod. The M_1 plants were harvested individually and the seeds obtained were used to raise the M_2 generation as plant-to-row progenies. Chlorophyll mutations were scored immediately after the emergence of cotyledons in M_2 population.

RESULTS AND DISCUSSION

Studies in M_1 generation

The physical damage in the M_1 generation, caused by the mutagenic treatments can be determined by cytological analysis and by measuring plant injury. Plant injury can be measured by taking: Seedling height (determined 10-14 days after), Root length (determined soon after germination in the laboratory), Emergence under field or laboratory condition, Number of pods per plant, Number of seeds per pod and seeds per plant. According to (Gaul, 1970) the effects of physical and chemical mutagen on gene and chromosomal mutations in the biological material could be measured quantitatively by degree of reduction in germination, seedling survival, growth and fertility. Determination of M_1 injury using seedling height and survival is a routine procedure in mutation breeding, because it has been established that these characters are correlated with M_1 mutation frequency (Etsuo Amano, 2004).

In M_1 generation effect of mutagenic treatments was assessed on the basis of parameters survival (%), plant height reduction and seed sterility (in terms of pods/plant, seeds/plant and seeds/pod) against control. Among gamma rays and EMS combination treatments, higher dose of 100Gy +0.4% EMS was more effective, that induced 36.4 % reduction in plant height and 29.2% seed sterility (Table 1). Similar results were obtained in soybean by Patil *et al.,* (2004). In soybean, plant height was also found to be significantly reduced at higher doses of mutagenic treatment (Kumar and Rai, 2007). The highest concentrations of chemical mutagens inhibited the morphological development of seeds and seed viability reported by Nikolay *et al.,* (2005) in *Phaseolus vulgaris* seeds in M_1 generation. Wei *et al.,* (2008) reported that gamma rays had significant effect on the growth of M_1 plants, leading to significant reduction of fertility, seed set and plant height in rice.

Among gamma rays and SA combination treatments 50 Gy + 0.005% SA caused more damage. However among EMS treatment to 12 hrs, 16 hrs and 20 hrs water soaked seeds, Injury was less in case of 12 hrs presoaked compared to 16 hrs and 20hrs presoaked seed treatment may be due to active repair during this phases. EMS treatment to 16 hrs presoaked seeds exerted more injurious effects in terms of survival (%), seed sterility and plant height reduction (Table 1). The reduction in plant height and survival may be attributed to a drop in the auxin level (Gordon & Webber, 1955), chromosomal aberrations (Sparrow, 1961) or due to decline of assimilation mechanism (Quastler & Baer, 1950). Overall compare to all combination treatments, treatments of gamma rays and sodium azide (50 Gy + 0.005% SA) exhibited maximum seed sterility (33.3%) while 100Gy +0.4% EMS was exhibited maximum reduction in height (36.4%) and pod /plant (35.3) (Table 1).

Table 1: Effect of mutagenic treatments in M_1 parameters /generation

Treatment/ Dose	Seeds Sown	No. of Plants Survived	Survival (%)	Plant Height (cm)	Height Reduction (%)	Sterility		
						No. of Pod/plant	Total No. of seeds	Seeds/pod
Control	200	191	95.50	47.2	0.0	49.3	118.3	2.40±0.9 (00.0)
Gamma rays + EMS								
50 Gy + 0.2 %	800	514	64.25	$41.8^{\#}$	11.4	44.3	85.05	1.92±0.5 (20.0)
50 Gy + 0.4 %	800	425	53.13	$35.6^{\#\#}$	24.6	$36.3^{\#\#\#}$	63.50	1.75±0.8 (27.1)
100 Gy + 0.2 %	800	490	61.25	$41.2^{\#\#}$	12.7	43.5	82.65	1.90±0.7 (20.8)
100 Gy + 0.4 %	800	466	58.25	$30.0^{\#\#\#}$	36.4	$35.3^{\#\#\#}$	60.01	1.70±0.6 (29.2)
Gamma rays + SA								
50 Gy + 0.003%	175	83	47.43	44.5	5.7	44.7	85.37	1.91±0.7 (20.4)
50 Gy + 0.005%	175	48	27.43	$38.4^{\#\#\#}$	18.6	42.4	67.84	1.60±0.5 (33.3)
PSW+ EMS								
12hrs + 0.8%	225	90	40.00	$37.5^{\#\#\#}$	20.6	$42.0^{\#}$	82.32	1.96±0.7 (18.3)
16hrs + 0.8%	225	82	36.44	$33.5^{\#\#\#}$	29.0	$40.5^{\#\#}$	77.35	1.91±0.7 (20.4)
20hrs + 0.8%	225	83	36.88	$33.7^{\#\#\#}$	28.6	$41.8^{\#}$	80.25	1.92±0.6 (20.0)

Parenthesis: % seed sterility; $^{\#, \#\#, \#\#\#}$ Significantly lower than control at probabilities of 0.05, 0.01 and 0.001 respectively. Values represents mean ± SD.

In this study, most of the treatments show more injurious effects at higher dosage. Constantin *et al., (*1976) observed decrease in survival, plant height and seed yield (sterility) with increase in dose rate of mutagen.

Studies in M_2 generation

Frequency and spectrum of chlorophyll mutations: Five types of chlorophyll mutants were recorded such as albina, xantha, chlorina, viridis and sectorials (chimera) (Table 3). Earlier such types of chlorophyll mutants were also observed in soybean

(Karthika and Subha Lakshmi 2006, Manjaya and Nandanwar 2007).

All the mutagenic treatments induced chlorophyll mutations. Among gamma rays and EMS combination treatments 50Gy +0.4% EMS and 100Gy +0.4% EMS induced broader spectrum. The range in the chlorophyll mutation frequency recorded was from 0.49-2.91% on M_2 plant basis (Table 2) among combination treatments of gam ma rays and EMS. The treatment 100Gy +0.4% EMS was highly effective since it induced maximum frequency (2.91%) on M_2 plant and M_1 family (27.25%) bases (Table 2). This also induced maximum spectrum of chlorophyll mutations that included all the five mutants.

Table 2: Frequency of induced chlorophyll mutations in the M_2 generation

Treatment Dose	Population size		Mutated	Total chlorophyll	Frequency of mutants/100	
	M_1 families	M_2 plants	M_1 families	mutant No	M_1 families	M_2 plants
Control	191	3812	0	0	00.00	0.00
Gamma rays + EMS						
50 Gy + 0.2 %	514	11,168	70	86	13.61	0.77
50 Gy + 0.4 %	425	5,948	82	122	19.29	2.05
100 Gy + 0.2 %	490	12,039	39	59	07.95	0.49
100 Gy + 0.4 %	466	7,890	127	230	27.25	2.91
Overall	**1895**	**37,045**	**318**	**497**	**16.78**	**1.34**
Gamma rays + SA						
50 Gy + 0.003%	83	2,735	2	4	2.40	0.15
50 Gy + 0.005%	48	1,986	2	4	4.16	0.20
Overall	**131**	**4,721**	**4**	**8**	**3.05**	**0.17**
PSW+ EMS						
12hrs + 0.8%	90	1,165	27	44	30.00	3.78
16hrs + 0.8%	82	1,023	25	46	30.50	4.50
20hrs + 0.8%	83	965	15	22	18.10	2.28
Overall	**255**	**3,153**	**67**	**112**	**26.27**	**3.55**

Table 3: Spectrum of induced chlorophyll mutations in the M_2 generation

Treatment Dose	M_2 plants	Total chlorophyll mutant No.	Frequency of mutants/100 M_2 plants									
			Albina		Xantha		Chlorina		Viridis		Chimera	
			No.	%	No.	%	No.	%	No.	%	No.	%
Control	3812	0	0	0.0	00	00.0	00	00.0	00	00.0	0	0.0
Gamma rays + EMS												
50 Gy + 0.2 %	11,168	86	0	0.0	13	15.1	17	19.8	56	65.1	0	0.0
50 Gy + 0.4 %	5,948	122	2	1.7	14	11.7	45	37.5	59	49.2	2	1.7
100 Gy + 0.2 %	12,039	59	0	0.0	09	16.0	16	28.6	31	55.4	3	5.3
100 Gy + 0.4 %	7,890	230	17	7.5	36	15.9	68	29.6	106	46.6	3	1.3
Overall	**37,045**	**497**	19	**3.9**	**72**	**14.7**	**146**	**29.6**	**252**	**51.5**	**8**	**1.6**
Gamma rays + SA												
50 Gy + 0.003%	2,735	4	0	0.0	0	0.0	2	50.0	2	50.0	0	0.0
50 Gy + 0.005%	1,986	4	0	0.0	0	0.0	2	50.0	2	50.0	0	0.0
Overall	**4,721**	**8**	**0**	**0.0**	**0**	**0.0**	**4**	**50.0**	**4**	**50.0**	**0**	**0.0**
PSW+ EMS												
12hrs + 0.8%	1,165	44	2	4.5	16	36.4	11	25.0	15	34.1	**0**	**0.0**
16hrs + 0.8%	1,023	46	1	2.2	6	13.0	13	28.3	26	56.5	**0**	**0.0**
20hrs + 0.8%	965	22	2	9.1	5	22.7	7	31.8	8	36.4	**0**	**0.0**
Overall	**3,153**	**112**	**5**	**4.5**	**27**	**24.1**	**31**	**27.7**	**49**	**43.7**	**0**	**0.0**

Among 50 Gy+ 0.003 % SA and 50 Gy+ 0.005% SA the latter was more effective. However all the types of mutants were not observed in these treatments, only chlorina and viridis were obtained and albino, xantha and chimera were totally absent (Table 3). Although among two, 50 Gy+ 0.005 % SA was better the yield of mutants was very low (0.2) on the M_2 plant and on M_1 plant basis (4.16).

EMS treatment to presoaked seeds induced broad spectrum of mutations including all the four. The range of chlorophyll mutation frequencies estimated in these treatments was between 2.28 and 4.50%. Among presoaking condition EMS treatment to 16 hrs presoaked seeds induced maximum frequencies on M_2 plant (4.50) and on M_1 family (30.5) bases.

Among all the mutagenic treatments used here the range of chlorophyll mutation frequencies recorded was from 0.15 - 4.50%. The treatment EMS 0.8% to 16hrs presoaked seeds was the most effective one (Table 4.2).

The frequency of chlorophyll mutations is considered as a dependable index for evaluating the efficiency and effectiveness of the mutagens and their effective concentration, so as to use them in mutation breeding (Gustafsson, 1951).

Among different chlorophyll mutants viridis appeared more than others in all the treatments employed. The reason for the appearance of greater number of viridis type may be involvement of polygenes in chlorophyll formation (John Ahmed 1996).

The albina mutants were found only at higher doses in combination treatment of gamma rays (50 and 100 Gy) with 0.4% EMS. Among EMS treatment to presoaked seeds albinas were also recorded but among gamma rays+SA treatment albina and xantha were totally absent. According to Lakshmi *et al.,* (1996), sodium azide stimulates the synthesis of chlorophylls and proteins at lower concentration and enhance the biochemical products in practical mutation breeding programme.

Albina and xantha did not survive longer and died early. In the study of Noble *et al.,* (1977) achlorophyllous lethal yellow soybean mutant was capable of survival when provided with a suitable carbon source. Viridis mutants survived till the maturity. The presence and absence of some chlorophyll mutants in some mutagenic treatments are indicative of differences in the availability of the loci to mutagen.

In the present work frequency of chlorophyll mutations increased with the higher concentration of EMS (0.4%) along with 50 and 100 Gy gamma rays dose in combination treatment. The increase was also observed at the higher dose of SA in combination with gamma rays. In the present study among the EMS treatments to pre-soaked seeds the frequency reached maximum at 16 hrs presoaking. EMS treatment to presoaked seeds are more effective due to high permeability and among the presoaked hours a particular duration (16 hrs) in the present study can be attributed to availability of maximum sites available to EMS treatment may be due to maximum peak of DNA synthesis (Khalatkar, 1977) .

In the present study EMS treatments to presoaked seeds exhibited highest chlorophyll mutation frequency than others combination treatments in the M_2 generation. Karthika and Subha Lakshmi (2006) also found EMS to be highly superior to gamma rays in inducing higher frequencies and wide spectrum of chlorophyll mutations in soybean. Several workers reported higher chlorophyll mutation frequencies due to EMS treatments in different crops, in mungbean with EMS>gamma rays (Singh *et al.,* 2000).

Ryan and Heslot (1963) stated that induction of chlorophyll mutations is dependent on the randomized action of physical mutagens, whereas, the EMS has specificity to certain loci in barley. As shown in barley chlorophyll mutations depend on the number of loci getting affected by mutagen (Nilan 1966), since chlorophyll development seems to be controlled by many genes located on several chromosomes (Natarajan and Upadhya, 1964).

In the present study only two plants with yellow green patches on leaves (chimeric mutants) were observed in the M_2 population of 100 gy + 0.4% EMS treatment. Leaf variegated mutants of soybean affecting chloroplast development can arise through either nuclear or cytoplasmic mutation events (Honeycutt *et al.,* 1990), small or large green sectors have been found within large sectors of yellow tissue suggesting the possibility that somatic reversions from a recessive form (yellow leaf sectors) to a dominant form (green leaf sectors) may occur. Yellow sectors, very light green sectors, and light green sectors can appear within sectors of green tissue. Palmer *et al.,* (2000) reported that the chimeric plants are a source of nuclear and cytoplasmically inherited mutants.

The origin of cholorophyll deficiency is mainly due to mutations in genes, which are responsible for synthesis of photosynthetic pigments. Benedict (1972) reported that the chlorophyll deficient mutants lack the well-defined grana structure of the chloroplasts. A possible mechanism to explain the chlorophyll abnormalities has been suggested on the basis of the decreases in malate dehydrogenase (MDH) activity (Elmer *et al.,* 1994).

Conclusion

Obtained results conclude that the combination of mutagens are more effective than individual treatment. Among gamma rays and EMS combination treatments, higher dose of 100Gy +0.4% EMS was more effective, that induced maximum % chlorophyll mutations. Frequency of chlorophyll mutations increased with the higher concentration of EMS (0.4%) along with 50 and 100 Gy gamma rays dose in combination treatment. Viridis appear in maximum % and albina in less percentage in all treatments over other types of chlorophyll mutations. Present work will help to find out type and effective concentration of mutagen for the mutation breeding in Soybean.

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