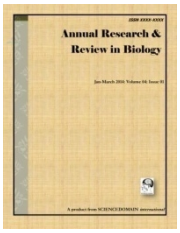

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Mutagenic Effects of MH and MMS on Induction of Variability in Broad Bean (*Vicia faba* L.)

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Authors' contributions

This work was carried out under supervision of author SK. Author SK designed the study, wrote the protocol and author RAL performed the experiments, observations, statistical analysis, the literature searches and wrote the manuscript. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aims: An experiment was conducted to find out the comparative response of MMS and MH on *Vicia faba* L. var. Nepal Selection with a view to determine the mutagen and treatment causing maximum bio-physiological and cyto-morphological variation as compared to the control for genetic improvement of crop.
Study Design: Induced mutation breeding study.
Place and Duration of Study: Mutation Breeding Laboratory, Department of Botany, Aligarh Muslim University, Aliagrh, during Rabi seasons of 2010 to 2012.
Methodology: The dry and healthy seeds of uniform size were treated with four concentrations viz, 0.01%, 0.02%, 0.03%, 0.04% of the mutagens (MH and MMS) independently for 6 hours. The observations were made on seed germination, seedling height, total chlorophyll content, morphological variation in leaf and flower arrangement, pollen fertility in M_1 generations and quantitative traits such as days to maturity, plant height, leaves per plant, pods per plant, seeds per plant, 100 seed weight (g) in M_2 generations.
Results: The results of the present investigation clearly revealed that MH and MMS both induced similar type of mutation in broad bean but extent and frequency of variation greatly varies. Biological damages namely reduction in seed germination, seedling height and

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pollen fertility were more in 0.03% and 0.04% MH compare to MMS and also total chlorophyll content was lowest at 0.04% MH. The recorded inhibition percentage shows the higher effectiveness of MH compare to MMS in inducing mutation in M_1 population. Frequency of morphological variations and cytological aberrations was observed to be more in higher doses of MH than MMS. Further in M_2 generation, early maturing mutants were obtained from 0.01% & 0.02% MH and 0.02% & 0.03% MMS treated plants. Plant height and leaves per plant decreases with increasing doses but 0.04% MH treatment gives dwarf variants with reduced height with few small leathery leaves. Yield and attributing traits showed positive mutation at 0.01%, 0.02% & 0.03% MMS compared to control.

Conclusion: Lower or intermediate doses of both the mutagens were found to be useful to improve the genetic background of broad bean, especially in seed yield and early maturity. MH and MMS both induced similar type of variation but degree and frequency of variation is more in MH treated populations in all the parameters studied in *Vicia faba* L.

Keywords: Vicia faba L.; maleic hydrazide (MH); methylmethane sulphonate (MMS); Induced variation; mutation breeding.

1. INTRODUCTION

Pulse crops play an important role in the diets of poor people around the world and complement cereal crops as a source of protein and minerals while agronomically they serve as rotation crop with cereals, reducing soil pathogens and supplying nitrogen to the cereal crop. Broad bean (*Vicia faba* L.) is one of the oldest crops grown by humans and is a valuable protein-rich food and animal feed in developing countries [1]. Cultivated broad bean is used as a vegetable and is considered in some areas to be superior to other legumes and as a meat and skim-milk substitute.

Induced mutation is the ultimate source to alter the genetics of crop plants that may be difficult to bring through cross breeding and other breeding procedures. Therefore, during the last several years, different mutagens have been used by various workers to induce genetic variability in various crop plants [2-10]. Genetic variability has been induced through mutagenesis in several pulse crops using MH and MMS synergistically with other mutagens, but the information available about their independent efficiency and effectiveness on *Vicia faba* is meager, so induced mutations were undertaken to bring-forth genetic improvement of this plant and to estimates its sensitivity towards these mutagens. In the present study attempt has been made to understand the effectiveness of MMS (a monofunctional alkylating agent) and MH (a promutagen activated into mutagen in plants highly likely by peroxidase) on *Vicia faba* L. on the induction of mutation to find out the mutagen doses producing useful variation.

2. MATERIAL AND METHODS

The experimental plant material used in the present investigation is commercial variety of broad bean (*Vicia faba* L.) var. Nepal Selection. Seeds presoaked in distilled water for 9 hours, were treated with different concentrations of MMS (v/v) and MH (w/v) viz. 0.01%, 0.02%, 0.03% and 0.04% prepared in sodium phosphate buffer at 7.0pH for 6 hours at room temperature. Thoroughly washed 270 seeds were sown in three replications for each treatment of the mutagen used as well as untreated to served as control in earthen pots filled with soil manure and kept in the Net House of the Department of Botany, Aligarh Muslim

University (Aligarh), to raise M_1 generation during winter season of 2010-11. Seed germination and seedling height (root and shoot lengths) were calculated after 10 days of sowing in petri dishes kept in the B.O.D. incubator at 27±1ºC temperature. The chlorophyll contents of leaves were estimated by the method of MacKinney [11]. Pollen sterility was determined from 20 randomly selected plants per treatment, along with control. The pollen grains from freshly dehisced anthers were stained with 1% acetocarmine. Pollen grains stained as uniform deep red colours were counted as fertile and others as sterile. Morphological variation in leaf and flower arrangement and pod type were recorded at the time of maturity. Meiotic studies were conducted with 1% acetocarmine squash technique using young flower buds of the 20-25 selected M_1 variants which was previously fixed and stored in Carnoy's fluid and 70% alcohol respectively. Seeds of the selected M_1 plants were sown in pots to raise M_2 generation in the crop season of 2011-12. Statistical data on quantitative traits were calculated from the mature M_2 population. Mean ($\bar{X}$), Standard deviation (S.D.) and coefficient of variation (C.V.%) were calculated to determine the degree of intra and inter-population variation induced and to compare the impact of different doses of mutagens on different traits.

3. RESULTS AND DISCUSSION

In the present investigation, various viable bio-physiological and cyto-morphological variants showing a wide range of variability were recorded in the M_1 and M_2 generations of the mutagens treated populations. The result of the present investigation clearly revealed that MH and MMS both induced similar type of variation. It was earlier observed that the action of MH on chromosomes has been described to be very similar to those of alkylating agents [12]. The values of the dose response for MH and MMS in results have confirmed that variation is noteworthy in MH treated populations of *Vicia faba* in all the parameters studied. Induction of mutations similar to the results of the present study by different mutagens in *Vicia faba* has also been reported by several workers [13-22].

3.1 Biological Damages

Percentage inhibition in seed germination was highest 70.59 and 29.41 per cent with 0.04% MH and 0.04% MMS, respectively. Data recorded on biological damage showed all treatments of both the mutagens brought about reduction in seed germination, seedling height and pollen fertility. Seedling length decreases from 5.33cm (control) to 3.14 cm at 0.04% MMS and 2.08 cm at 0.04% MH. The pollen fertility was highest in control and the lowest percentage of pollen fertility was found in 0.04% of MH treatment (Table 1). Similar result was also reported in *Vicia faba* [23] and in *Vigna radiate* [24]. The reduction in germination may be due to destruction of the activity of gibberellic acid, following the radiation treatment [25] and metabolic disturbances during germination [26]. It was also considered that reduction in germination percentage was due to weakening and disturbances of growth process, regulated in early elimination of seedlings [27,28]. The inhibition of germination may be due to interaction between mutagen and the seed cell system [29]. It may also be due to toxicity of mutagens followed by mutational changes at genic or chromosomal level because the reduction in germination corresponds with the increasing chromosomal aberrations.

The pollen fertility was highest in control and the lowest percentage of pollen fertility was found in 0.04% of MH treatment (Table 1). The aberrant pollen grains due to vast array of meiotic aberrations, point mutation or probably invisible deficiencies led to pollen sterility at

higher concentrations of mutagen. The dose dependent pollen sterility with increase in mutagenic concentration was also observed in *Vicia faba* [14] and in *Vigna radiate* [24].

3.2 Meiotic Studies in M_1 Generation

Chromosomal abnormalities were studied at the different stages of meiotic division. No such abnormalities were observed in pollen mother cells (PMCs) of control plants. However, in the treated plants the most frequent aberrations were recorded like unsynchronized separation of chromosomes, chromatin bridges, stickiness of chromosomes, micronuclei, disturbed polarity at anaphase and telophases which were present at the higher treatments of mutagens (Fig. 1). The impact of MH and MMS for creating biological damages in terms of meiotic aberration and pollen fertility in M_1 generation of *Vicia faba* was assessed and it revealed that the sensitivity of present biological system towards MH is much higher than MMS. Stickiness at metaphase I and anaphase I among the chromosomes might have occurred due to disturbances of cytochemically balanced reactions by the effects of mutagens [30]. Bridges at Anaphase I and II could be due to delayed terminalisation, stickiness of chromosome ends, failure of chromosome movement, late terminalisation and unequal separation of chromosomes [31]. Disturbed polarity in *Vicia faba* was also reported due to spindle disturbances [32]. Meiotic abnormalities observed may be due to toxic effects of mutagens which might have created an error in DNA repair mechanism and in cell division. The differences in the degree of induced chromosomal abnormalities may be related to the differences in the efficiencies as well as mechanism of action of mutagens [33].

3.3 Morphological Variants

In control plants, the leaves were pinnately compound had 2-4 leaflets which were obovate, entire, acute, and smooth. Different types of morphological variations have been recorded in treated populations, such as incomplete fusion of two leaflets forming a heart like shape with slight notching. Some leaflets were rudimentary, thick, and leathery. In higher concentrations all seedlings showed stunted growth bearing small, dark green and thick leaflets. In 0.04% MH the entire leaflets were highly reduced in size, round, deshaped and rudimentary and lanceolate also (Fig. 2. i-vi). The flower arrangement on the control plant were born four, however, in the moderate dose double flower and triple flower were also noticed. In the high doses the flower number gets reduced (Fig. 2. xii-xv). In moderate doses few Bushy variants characterized by increase in branching with thicker internodes and greater number of nodes and consequently increase in number of fruits and seeds were observed in comparison to control and higher treatments (Fig. 2: xvi-xviii). Alteration in pod sizes viz; bold pod, narrow pods have also been recorded. Normally in moderate dose the pod sizes were seen larger more frequently than control and in higher dose the size of pod gets shrunken and reduced (Fig. 2. xix). The leaf abnormalities may also be due to actual mutation processes which are most easily induced in leguminous plants [34] or due to chromosomal alterations [35] or due to the number of genes with pleiotrophic effect [36].

Table 1. Comparative effect of mutagens (MH and MMS) on seed germination, seedling height, pollen fertility and total chlorophyll content in *Vicia faba* L. var. Nepal selection in M_1 population

Treatments		Seed germination		Seedling height		Pollen fertility		Total Chlorophyll	
	Mutagens	Actual (%) Shift in mean	% age inhibition	Actual (cm) ± S.E Shift in mean	% age injury	Actual (%) Shift in mean	% age inhibition	Actual (mg/g) Shift in mean	% age inhibition
	Control	**94.44**	**--**	**5.33 ± 0.20**	**--**	**97.23**	**--**	**5.99**	**--**
0.01 %	MH	88.88-5.56	5.88	3.57 ± 0.60-1.76	33.02	82.56-14.67	15.09	5.09-0.90	15.06
	MMS	83.33-11.11	11.77	4.69 ± 0.58-0.64	12.01	89.08-8.15	8.38	5.12-0.88	14.72
0.02 %	MH	77.77-16.67	17.65	3.27 ± 0.55-2.06	38.65	76.73-20.50	21.08	4.28-1.71	28.60
	MMS	77.77-16.67	17.65	3.65 ± 0.57-1.68	31.52	85.33-11.90	12.24	4.69-1.30	21.74
0.03 %	MH	66.66-27.78	29.41	2.74 ± 0.50-2.59	48.59	67.27-29.96	30.81	4.04-1.95	32.61
	MMS	72.22-22.22	23.53	3.15 ± 0.62-2.18	40.91	81.53-15.70	16.14	3.85-2.14	35.79
0.04 %	MH	27.77-66.67	70.59	2.08 ± 0.49-3.25	60.97	56.80-40.43	41.58	2.28-3.71	61.94
	MMS	66.66-27.78	29.41	3.14 ± 0.30-2.19	41.09	78.66-18.57	19.10	4.46-1.53	25.59

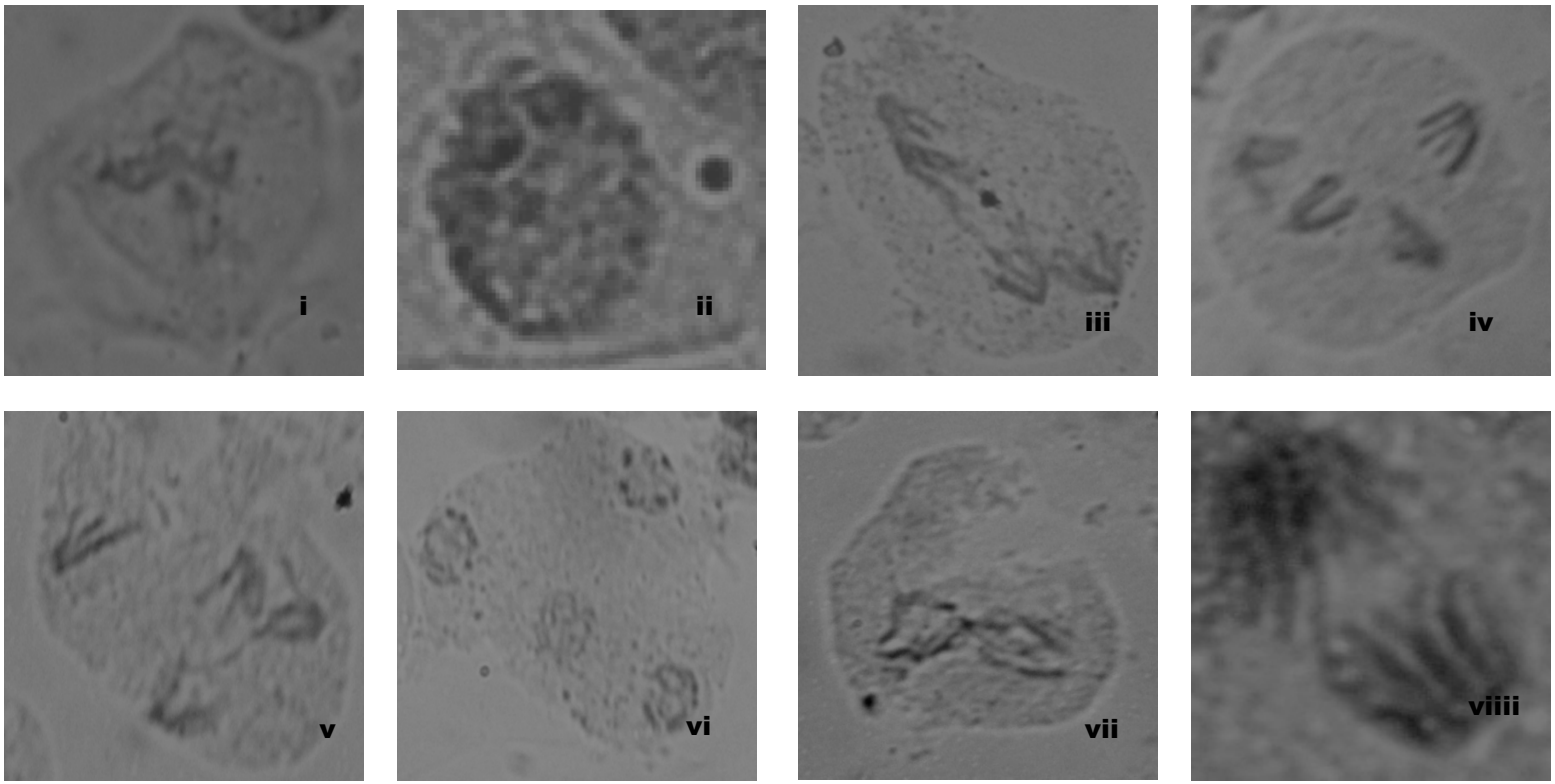

Fig. 1. Meiotic cell division stages i- Stickiness at Metaphase-I; ii- Micronuclei; iii- Anaphase I showing chromosomal bridge; iv- Anaphase II showing abnormal polarity; v- Anaphase II showing chromosomal bridge; vi- Telophase-II showing disturbed polarity; vii- Metaphase I with abnormality; viii- Stickiness at Anaphase I.

3.4 Leaf Chlorophyll Variants and Content

It was observed that total chlorophyll content of leaves ranged from 5.99 mg/g in the control to 2.28 mg/g in 0.04% MH and 3.85 mg in 0.03% MMS (Table 1). In the present study, the chlorophyll variants were scored from 7 to 10 days after sowing. Two different types of chlorophyll variants viz. chlorina and xantha were recorded in the pot experiments (Figure 2: vi & viii). Of the two mutagens, MH showed the higher number of chlorophyll variants. Occurrence of xantha and chlorina mutants in large number of crops have been attributed to different causes such as impaired chlorophyll biosynthesis, further degradation of chlorophyll and bleaching due to deficiency of carotenoids [37]. It should also be stressed that colour of the leaves of certain cultivars and varieties is not always directly correlated with chlorophyll concentration [38]. The high content of chlorophyll was considerably dropped at higher concentrations of mutagen. The reason behind variability in total chlorophyll content may be the mutation at various loci of genome by various concentrations of mutagens [39].

Fig. 2. Note: i-vi represent isolated morphological variants: control plant with pinnately compound leaf had 4 leaflets which were entire, acute, and smooth; obovate and entire; round apex; incomplete fusion of two leaflets forming with deep notching; heart like shape with slight notching; rudimentary, thick and leathery, respectively. vii & viii represent chlorina & xantha chlorophyll variants. ix-xi represent plant height reduced in comparison to the control; stunted growth bearing single flower and small, dark green and thick leaflets; dwarf mutant, respectively. xii-xv represents variation in number of flowers at the reproductive node from four in control to one. xvi-xviii represents bushy variants with thick and long internode , more nodes bearing healthy pods at moderate doses; relatively thinner and shorter internode; weak internode bearing few pods, respectively at higher doses. xix represent pods variant in control and mutagen treated plants showing different sizes.

3.5 Quantitative Characters

Data on quantitative characters are given in Table 2. Conspicuous differences in yield attributing traits were recorded in all the mutagenic treatments of MH and MMS. Lower doses of MMS showed positive variation while higher doses of MH proved lethal. The mean of days to maturity is maximum 122.03 days at 0.04% of MH and minimum 118.67 days at 0.03% MMS while it is 120 days for control. Some treatments of mutagen brought about a notable decrease in days to maturity. Height of mature plants generally decreased with increasing concentrations of both the mutagens. In 0.04% MH, plant height reduced considerably giving dwarf mutants and some immature sterile plant (Fig. 2: ix-xi). The average number of leaves in control was 39.50 per plant whereas it decreased gradually with increasing concentration of mutagens to 15.16 in 0.04% MH. Mean value for pods per plant was 6.18 in control and it ranged from 6.94 to 3.60 in MH and 8.00 to 6.00 in MMS treated population. An average 21.94 seeds per plant was recorded in control and 21.86 to 8.46 in MH and 26.16 to 16.98 in MMS treated population. Weight of 100 seeds in control was 55.7 g. It decreased in all the concentrations of mutagens in M_2 generation from 51.7 g to 40.1 g in MMS and 44.9 g to 31.5 g in MH. Variation in quantitative traits have been attributed to the structural changes in the constitution of chromosomes or chromosomal damage [40] or may arise from interference of mutagens with the cell elongation [41] or injury caused to the meristematic cells [42]. Some other aspects such as auxin reduction, physiological disorder or metabolic disturbances have also been reported. Generally meiotic abnormalities are the cause for pollen sterility [43,44]. The physiological and chromosomal disturbances in growth process may also play important role, because quantitative traits are also controlled by protein synthesis regulated under the direction DNA. The quantitative traits showed intra-population deviation from their mean values as exhibited by high S.D. and C.V. in most cases.

Table 2. Effect of mutagens (MH and MMS) on Mean ($\bar{X}$), shift in mean, S.D. and C.V. of various quantitative characters in *Vicia faba* L. var. Nepal Selection in M_2 generation

Treatments	Mutagens	Quantitative characters					
		Days to maturity Mean ± SD CV%, Shift in mean	Plant height Mean ± SD CV%, Shift in mean	leaves/plant Mean ± SD CV%, Shift in mean	Pods/plant Mean ± SD CV%, Shift in mean	Seeds/plant Mean ± SD CV%, Shift in mean	100 Seed Weight (g) Mean ± SD CV%, Shift in mean
	Control	**120.00 ± 0.18 1.68, ----**	**47.76 ± 3.79 7.95 ----**	**39.50 ± 2.68 6.94, ----**	**6.18 ± 1.28 20.71, ----**	**21.94 ± 1.59 20.93, -----**	**55.70 ± 0.33 0.59, ----**
0.01%	MH	119.07±0.22	37.25 ± 5.26	32.16 ± 4.25	6.94 ± 0.80	21.86 ± 0.86	44.90 ± 0.41
		1.87, -0.93	14.13,-10.51	13.12, -7.34	11.52, +0.76	9.11, -0.08	0.91, -10.80
	MMS	120.10±0.23	44.53 ± 4.76	34.33 ± 3.75	8.00 ± 1.15	26.16 ± 0.85	51.70 ± 0.25
		1.73, + 0.10	10.70, -3.23	9.69, -5.17	14.38, +1.82	16.15, +4.22	0.48, -4.00
0.02%	MH	117.87±0.23	36.36 ± 6.34	28.83 ± 5.33	6.21 ± 0.86	18.69 ± 1.28	44.40 ± 0.11
		1.92, -2.13	17.44, -11.40	16.43, -10.67	13.84, +0.03	15.44, -3.25	0.24, -11.30
	MMS	119.33 ±00.21	38.42 ± 6.22	29.83 ± 5.21	8.36 ± 1.11	25.75 ± 1.05	50.50 ± 0.28
		1.56, -0.67	16.18, -9.34	14.17, -9.67	13.28, +2.18	17.72, +3.81	0.55, -5.20
0.03%	MH	120.80±0.22	29.00 ± 9.85	24.50 ± 8.84	4.00 ± 0.87	11.16 ± 0.78	36.70 ± 0.48
		1.87, +0.80	33.96, -18.76	32.95, -15.00	21.75, -2.18	20.09, -10.78	1.31, -19.00
	MMS	118.67± 0.22	35.38 ± 8.27	18.50 ± 7.26	8.31± 1.26	24.52 ± 0.77	48.40 ± 0.37
		1.62, -1.33	23.37, -12.38	21.36, -21.00	15.16, +2.13	12.25, +2.58	0.76, -7.30
0.04%	MH	122.03 ± 0.22	14.20 ± 11.67	15.16 ± 10.66	3.60 ± 2.15	8.46 ± 0.95	31.50 ± 0.65
		1.92, +2.03	82.17, -33.56	52.16, -24.34	59.72, -2.58	56.05, -13.48	2.06, -24.20
	MMS	121.00 ± 0.27	31.33 ± 5.72	21.33 ± 4.71	6.00 ± 1.00	16.98 ± 0.69	40.10 ± 0.53
		2.00, +1.00	18.25, -16.43	17.24, -18.17	16.67, -0.18	19.35, -4.96	1.32, -15.60

Since the quantitative traits are governed by many genes, mean and coefficient of variation for six quantitative traits studied provide sufficient evidence that mutagenic treatments could alter mean values and create additional genetic variability. The treated series of data for which the coefficient of variation is large indicates that the group is more variable and therefore, proper screening of variants and their stabilization in future generation could be very useful.

4. CONCLUSION

The present pursuit therefore showed that in *Vicia faba*, a broad spectrum of mutations comprising of different types is induced by the MH and MMS which are concentration dose dependent and more or less linear and the performance of all the traits studied decreased in higher dose of mutagens. The combined analysis of the different parameters suggested that frequency of mutation was more in MH treated population. In conclusion, it is advocated that the lower or intermediate treatments of both MH and MMS were found effective in inducing sufficient genetic variability in the available *Vicia faba* L. genotypes for the isolation of early maturity with high yield and yield attributing trait mutants. The results will be exploited in future generation for stability of traits and establishing mutation breeding protocols for *Vicia faba* L..

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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