International Journal of Botany Studies
ISSN: 2455-541X; Impact Factor: RJIF 5.12
Received: 02-10-2020; Accepted: 17-10-2020: Published: 03-11-2020
www.botanyjournals.com
Volume 5; Issue 6; 2020; Page No. 37-42

Physio-morphological Variations induced by methyl methane Sulphonate in *Trigonella foenum-graecum* L

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Abstract
Since age-old times, herbal therapeutics has been involved in conventional systems of medicine such as Unani and Ayurveda. Mutation breeding has involved advance genetic approach and recognition of ameliorating or novel phenotypes and their incorporation into breeding programmes. Fenugreek (*Trigonella foenum-graecum* L.), an important medicinal herb of the family Fabaceae with therapeutic importance and was preferred in the present work to examine the response of potent chemical mutagen, i.e., Methyl Methane Sulphonate (MMS) on physio-morphological traits in M_1 generation. Germplasm (seeds) of two varieties of fenugreek was exposed to four mutagenic concentrations of MMS, i.e., 0.01%, 0.02%, 0.03%, and 0.04% contributing to the genetic dissection of the wild type genes. As the concentration of mutagen increased, it showed inhibitory effects on germination percentage, seedling height, plant survival, and pollen fertility. Total chlorophyll content exhibits both negative and positive shifts while negative correlation observed in carotenoid content from their control in both varieties. Scrutiny on quantitative attributes involving plant height, fertile branches/plant, number of pods/plant, number of seeds/pod and 1000 seed weight exhibit remarkable variations at different mutagenic treatments. This analysis revealed that lower and intermediate doses of mutagen exerted less biological damage and ameliorated the genotype of *Trigonella foenum-graecum*. Indeed, mutation facilitated crop breeding will play a pivotal role in the procreation of smart crop varieties and meet the challenges of the present and future exigency in medicinal crops.

Keywords: Induced mutagenesis, Methyl Methane Sulphonate (MMS), physio-morphological traits, *Trigonella foenum-graecum*

Introduction

Trigonella foenum-graecum is an annual herbaceous silage legume with aroma, indigenous to the Mediterranean region of the "old world" (Vavilov, 1926) [72], Asia (De Candolle, 1964) [19] and also originated in Turkey (Dangi *et al*., 2004) [18]. India contributes more than 68% of world production and the dominant country for fenugreek production (Petropoulos, 1973) [48]. It is a multipurpose cash crop utilize as herb, spice, and vegetable. Fenugreek is a key factor in health care both in the modern system and in Ayurvedic and conventional Chinese medicines. Fenugreek seeds and leaves have been exploited immensely for medicinal objectives (Basch *et al*., 2003) [11]. It is plenteous in polyphenolics that reduce oxidative haemolysis and inhibit peroxidation in human corpuscles (Rayyan *et al.*, 2010 [52], Belguith-Hadriche *et al*., 2013) [12]. In both animals as well as humans, Fenugreek inholds pharmacological attributes such as antidiabetic, anti-inflammatory, antioxidant, anticarcinogenic, hypocholesterolemic and neuroprotective effects (Yadav and Baquer, 2014) [75]. Nevertheless, their consumption in a desirable amount avert cancer (Raju *et al*., 2004) [50], lower cholesterol, and triglycerides concentration in the blood (Kamal-Eldin *et al*., 2000) [26] and regulate diabetes mellitus (Broca *et al*., 2000) [15]. Amin *et al*. (2005) [3] shown experimentally that fenugreek seeds defend breast cancer. Fenugreek is a valuable source of carbohydrates, proteins and lipids. In spite of this, contains alkaloids (trigonelline and choline), amino acids, glycosides (diosgenin), vitamin A, B_1, C, calcium, iron, and nicotinic acid (Newall *et al*., 1996 [45]; Budavari *et al*., 1996 [16]; Mehrafarin *et al*., 2010) [39]. Fenugreek is a diploid (2n=16), self-pollinated crop (Ahmad *et al*., 1999) [1] with an indeterminate growth habit bearing erect, branched stem, pinnate and trifoliate leaves, axillary yellowish-white flowers with yellowish-brown pods enclosing dull yellow to brown seeds. It has wide adaptability and tolerance to drought, frost and freezing weather. Fenugreek can be cultivated in a wide variety of soil. Hence, for increasing yield, a pH value of 6-7 should be maintained. Mutagenesis is an effective approach that generally enhances and uplifts the genetic makeup of plants. Additionally, seed mutagenesis has been employed for inducing chlorophyll mutations, morphological mutations, and yield parameters. The utmost purpose of mutation breeding is the advancement of genotypes showing amelioration regarding the current varieties. Further, the polygenic traits like early maturity, grain quality, grain yield, quality characters, biotic resistance, and abiotic stress have also been improved by mutagenesis (Kharkwal, 1996) [27]. Apart from conventional breeding techniques, mutagenesis provides a new departure for improving the plants in agriculture. Around 3222 mutant forms were out through induced mutations over the world for crop improvement. During the Second World War, the revelation of chemical mutagen was an additional landmark of induced mutations in the past. In mutation breeding, chemical mutagens develop into an inbuilt element for the development of mutants (Suchetana and Datta, 2012) [65]. Methyl methane sulphonate (MMS), an alkylating agent, and a powerful mutagen methylates deoxyguanosine at N7 and deoxyadenosine at N3. Alkylating agents cause depurination that creates a disparity in the DNA strand leading to frameshift of bases during duplication.

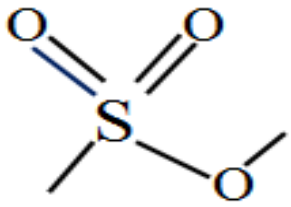


Fig1: Chemical structure of MMS ($C_2H_6O_3S$)

Materials and Methods

Vigorous, certified and viable seeds of two varieties *viz.* Desi methi and Metha of fenugreek were selected for the current investigation.

The seeds were mutagenized with different concentrations of mutagen, MMS, an alkylating agent. Stock solutions of methyl methane sulphonate (MMS) were made in phosphate buffer next to pH 7.0, maintaining the constant pH using buffer tablets. Five sets of viable seeds of each variety were isolated separately. A Single set of each one is maintained as control. Seeds were pre-soaked in distilled water for 12 hours, proceeded with treatments 0.01%, 0.02%, 0.03%, 0.04% MMS dosage level for 6 hours with intermittent shaking. Then, treated germplasm (seeds) were thoroughly rinsed by flowing tap water for the removal of leftover mutagen.

Seven seeds from every single treatment were raised upon moist cotton sited in petridishes to figure out the percentage of seed germination besides measuring the plantlet height, specifically root and shoot extent. Petri dishes were kept in the BOD incubator, maintaining 27±1ºC. Now, treated seeds of each treatment were sown in replicates, including untreated seeds in pots to elevate the M_1 variants. Consequently, morphological variants with distinct quantitative and qualitative traits from the treated together with untreated (control) populations were screened from the M_1 generation, which deviates from the control ones. The seed germination, plant survival percentage can be calculated by using the following formulae:

$$\text{Germination (\%)} = \frac{\text{Number of seeds germinated}}{\text{Number of seeds sown}} \times 100$$

$$\text{Survival (\%)} = \frac{\text{Number of plant at maturity}}{\text{Number of seeds germinated}} \times 100$$

Pollen fertility (%) was determined by collecting the fresh and mature flowers from the arbitrarily selected plants and staining the pollen grains by acetocarmine solution (2%). Pollen grains blemished and steady in outline deemed as fertile while others as sterile. It can be calculated by the following formulae:

$$\text{Pollen fertility (\%)} = \frac{\text{Number of fertile pollen}}{\text{Total number of pollen}} \times 100$$

Estimation of Chlorophyll and Carotenoid Content

The fresh leaves extraction for chlorophyll and carotenoid content (mg g^{-1} leaf fresh mass) measurement was made from 80% acetone and chlorophyll content can be calculated according to the Arnon's (1949) [7] proposed equation:

Total chlorophyll content =

$$[20.2\ (OD_{645}) + 8.02\ (OD_{663})] \frac{V}{1000 \times w}$$

And carotenoid content can be calculated by Kirk and Allen's (1965) [31] formulae:

Carotenoid content =

$$[7.6\ (OD_{480}) - 1.49\ (OD_{510})] \frac{V}{1000 \times w \times d}$$

Wherever,

OD_{645}, OD_{663}, OD_{480}, OD_{510} are the optical densities by 645, 663, 480 and 510 nm respectively; V, the volume of an extract; W, the mass of leaf tissues; d, the length of light path (1.4cm).

Statistical Assessment

Statistical evaluation was performed with SPSS 16.0 for windows. Analysis of Variance and Duncan multiple range test was executed amongst the treatments (p=0.05 significance level).

Results and discussion

Induced mutagenesis has a crucial value in lifting genetic makeup for crop amelioration by triggering micro mutations as well as visible macromutations for several agronomic traits that can be exploited in breeding techniques. For the last several years, various workers used different mutagens to stimulate genetic variability in various pulse crops for instance *Lens culinaris* (Sharma, 1990) [57], *Cicer arietinum* (Toker and Ilhan Cagirgan, 2004 [68]; Kozgar, 2014 [32]; Laskar *et al*., 2015) [38], *Vigna mungo* (Usharani and Kumar, 2015 [69]; Ramya *et al*., 2014) [51], *Vigna unguiculata* (Singh *et al*., 2002 [60]; Gnanamurthy and Dhanavel, 2014) [21], *Glycine max* (Patil *et al*., 2007) [47], *Vicia faba* (Laskar and Khan, 2014 [37]; Ahuja *et al*., 2014 [2]; Khursheed and Khan, 2017) [29], *Hordeum vulgare* (Khursheed and Khan, 2014 [28]; Khursheed *et al*., 2015) [30], *Vigna radiata* (Sangsiri *et al*., 2005 [53]; Wani *et al*., 2014) [73].

The present analysis examined the mutagenic outcomes of methyl methane sulphonate doses induced on various physio-morphological and quantitative parameters of *Trigonella foenum-graecum* L.

Seed germination, plant survival, and pollen fertility

In the present-day study, seed germination, survival, and pollen fertility exhibit reduction with enhancing level of mutagen. Similar outcomes were reported by Banu *et al*. (2005) [10] in cowpea and Amin *et al*. (2015) [4] in lentil. Noticeable variations observed were illustrated in Figure.2 A and 2B shows all the characters remarkably decreased with the increase in mutagen concentration. Germination percentage was recorded in control, inclusive of all mutagen treatments in under 1-6 days. The maximum percentage of seed germination was recorded in control of Desi methi and Metha, i.e., 96% and 90%, respectively. It decreased from 96% (control) to 74% (0.04% MMS) in Desi methi and 90% (control) to 70% (0.04% MMS) in Metha with the increasing concentration of mutagen. The germination may be suppressed possibly due to demolition of gibberellic acid activity, after the radiation treatment (Sideris *et al*., 1971) [58] as well as metabolic disorders throughout germination (Ananthaswamy *et al*., 1971) [5]. The germination rate reduction was owing to weakening combined with the disruption of the growth action leading to the elimination of premature seedlings (Griffiths and Johnston, 1962 [23]; Srivastava, 1979) [63].

The maximum plant survival occurred in controls (86% and 90%) of var. Desi methi along with var. Metha, respectively, falling off in highest dose of 0.04% MMS to 58% and 62% in var. Desi methi and var. Metha respectively. A gradual decline in the plant survival rate with an upsurge in the mutagenic concentrations of both physical as well as chemical was observed in *Lathyrus sativus* (Kumar, 1998 [35]; Kumar and Dubey, 1998) [34] and *Lycopersicon esculentum* (Jayabalan and Rao, 1987) [25]. The foremost cause for an extreme decrease in survival may be expected to physiological variation or chromosomal anomalies (Choudhary *et al*., 2012) [17]. Srivastava *et al*., (2011) [64] observed deminishing effect of mutagen on the seedling survival rate in wheat due to hindrance on several metabolic pathways of the cells. Comparable results have also been described by refs. In wheat by Rachovska and Dimova (2000) [49] and in sunflower by Mostafa (2011) [41].

The present analysis revealed that the pollen fertility was appreciably reduced (62.50% and 66.33%) in 0.04% of MMS for variety desi methi and Metha, respectively. The extent of percentage inhibition differed in different mutagenic treatments of both the cultivars (Figure.3). The degree of pollen sterility was observed high in var. Desi methi than var. Metha (Figure.4). The extent of sterility intensified with a rise in doses of mutagens in each of the variety of fenugreek. Goyal and Khan (2010) [22] observed the influence of methyl methane sulphonate (MMS) upon pollen fertility in urdbean (var. T-9). It was due to aberrant pollen grains as a result of chromosomal aberrations, point mutation or possibly microscopic deficiencies led to pollen sterility at higher doses of mutagen. The drop in pollen fertility with a rise in the level of mutagens doses may be ascribed to an increase in meiotic irregularities in addition to physiological disturbances. Similar findings were made by other workers (Muthusamy *et al*., 2005 [42]; Mensah *et al*., 2007) [40]. Pollen grains of mutated plants indicated germination inhibition upon various treatments. The RNA, protein as well as insignificant bioactive molecules in mature pollen allocate prompt germination along with tube growth (Taylor and Helper, 1997) [66].

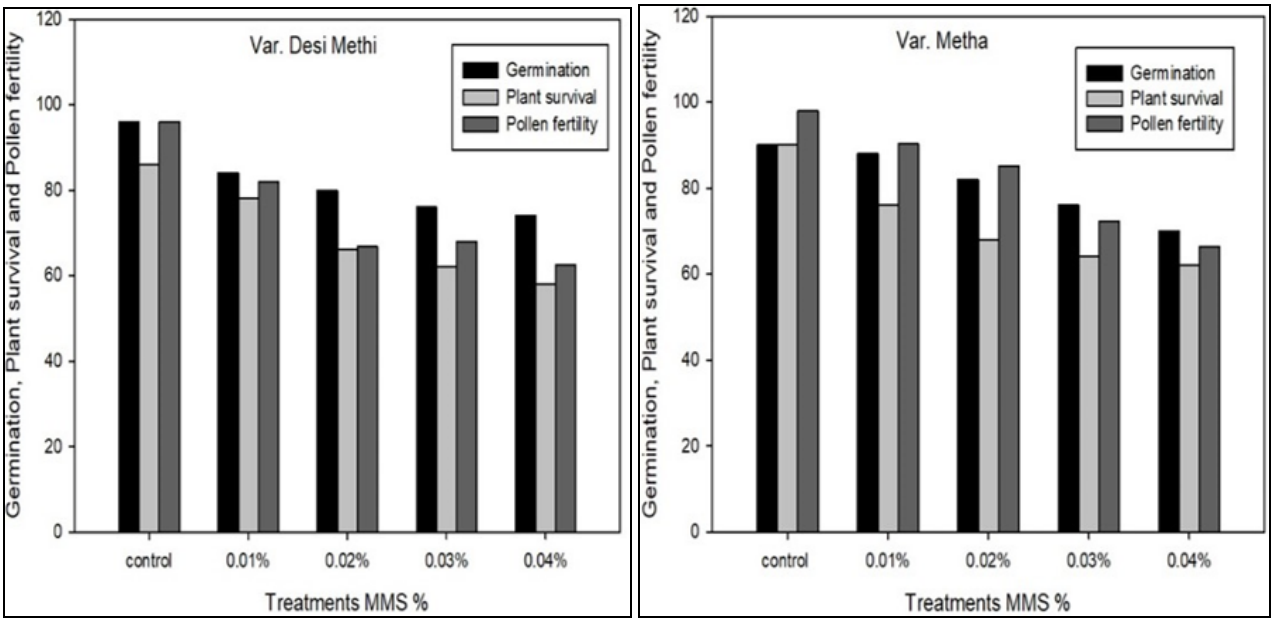


Fig 2: Impact of MMS upon germination, survival, and pollen fertility in M_1 generation (A) var. Desi methi (B) var. Metha of *Trigonella foenum-graecum* L.

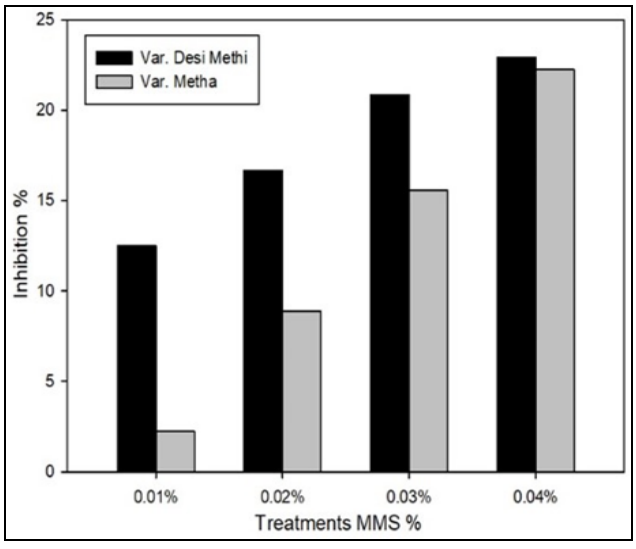


Fig 3: Percentage inhibition (%) in M_1 generation of (fenugreek) *Trigonella foenum-graecum* L. var. Desi methi, in addition to var. Metha.

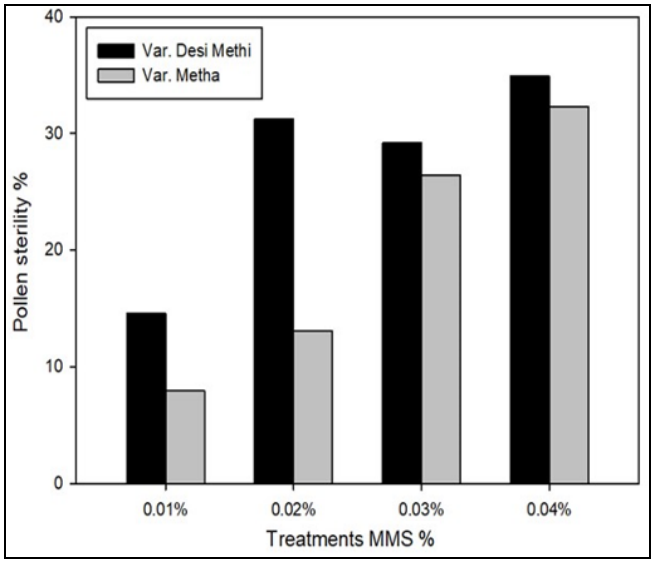


Fig 4: Pollen sterility (%) in M_1 generation of (fenugreek*)* *Trigonella foenum-graecum* L. var. Desi methi, in addition to var. Metha.

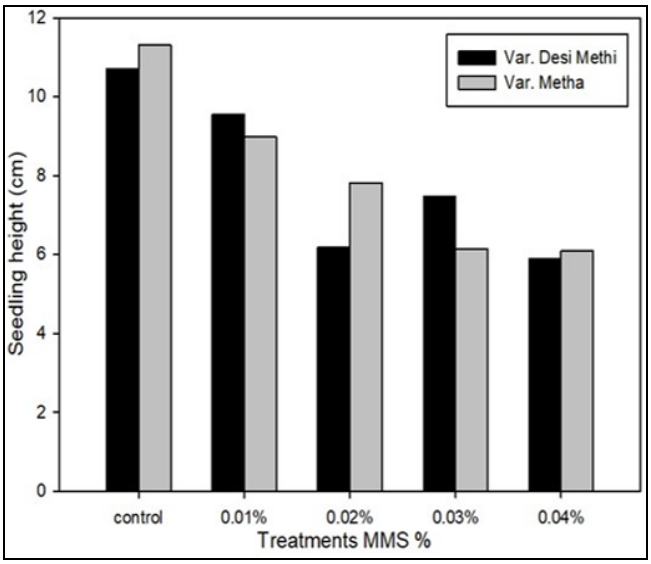


Fig 5: Seedling height (cm) in M_1 generation of (fenugreek) *Trigonellafoenum-graecum* L. var. Desi methi, in addition to var. Metha.

Chlorophyll and Carotenoid Content

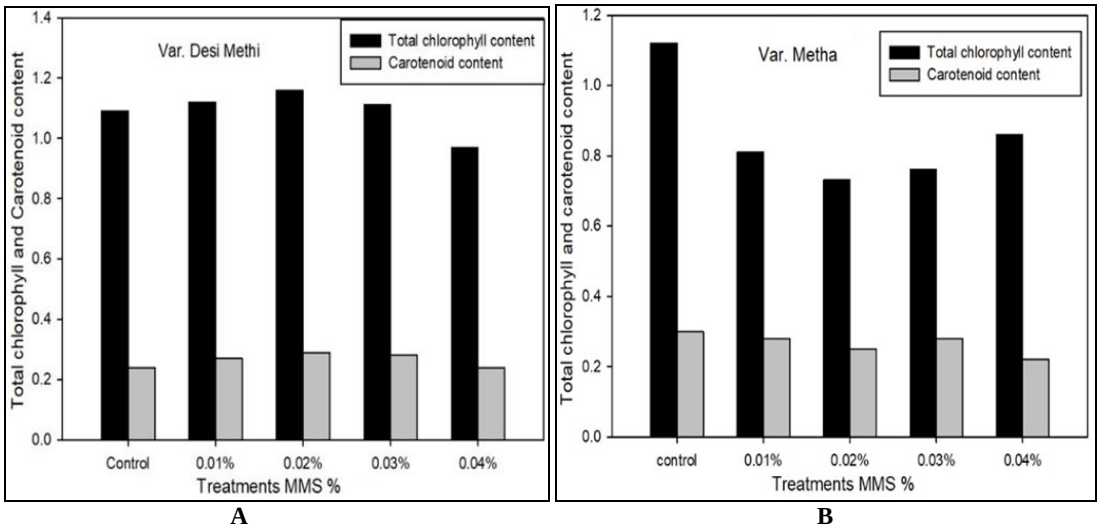
The figure 6A and 6B shows the total chlorophyll in control along with treated ones of the plant. Overall chlorophyll of leaves of one as well as the other, demonstrated both negative and positive change from the control at each of the treatments. In Desi methi, it remained 1.09 mg g^{-1} in control that decreased towards 0.97 mg g^{-1} in 0.04% MMS, while in Metha, it decreases from 1.12 mg g^{-1} (control) towards 0.86 mg g^{-1} (0.04% MMS).

The total carotenoid content also showed a negative shift from their parents. In desi methi, it was 0.24 mg g^{-1} in control which enhanced maximum to 0.29 mg g^{-1} in 0.02% MMS, while in Metha, it decreases from 0.30 mg g^{-1} (control) to 0.22 mg g^{-1} in 0.04% MMS.

Evaluation of chlorophyll and carotenoid of treated plants revealed a remarkable increase in contents due to the doses of the mutagenic treatments, which, to directs theimprove photosynthetic efficiency of the mutagenized population. Assessment of the chlorophyll content in the current analysis exhibit a wide-ranging discrepancy between treated populations through different mutagenic treatments. It was remarked that the total leaf chlorophyll content in each of the variety shows both positive and negative changes from their controls at all treatments. The total carotenoid content showed variable trend at all mutagenic treatments. Thus, it showed a negative shift from their parents in both the varieties.

Chlorophyll content is regarded as one of the indices of photosynthetic activity (Larcher, 1995) [36]. Chlorophyll enlargement appears to be operated by numerous genes situated on various chromosomes that can be adjoining to centromere in addition to proximal segments of chromosomes. Usually, chlorophyll content progressively declines from control towards a higher concentration of mutagen.

The prominent chlorophyll content at the beginning of the flowering phase, is supposed to take part in the organogenesis process (Simova *et al*., 2001) [59]. Nitrogen is a basic component of chlorophyll along with protein molecules, and its deficiency disturbs the chloroplast formation and chlorophyll accumulation in them (Saygideger *et al*., 2013) [54].



A **B**

Fig 6: Impact of MMS upon total chlorophyll (mg/g leaf fresh mass) and carotenoids (mg/g leaf fresh mass) in (A) var. Desi methi (B) var. Metha of *Trigonella foenum-graecton* L. in M_1 generation.

A B

Fig 7: Germination pattern and seedling height from mutagen treated seeds compared with control. A. Fenugreek seeds showing variations in germination patterns under different mutagenic doses after seven days of sowing in the laboratory. B. Fenugreek seeds showing seedlings height under different mutagenic doses after seven days of sowing in the laboratory.

Morphological Variants

Variations in morphology induced by MMS in both varieties of fenugreek is presented in the figure 8 and 9. The disparity in leaf morphology of plantlets and mature plants, such as unidentical, bifoliate, multilobed, partial chlorotic and undeveloped leaflets (Figure.8A-J) were frequently detected in superior doses. A plant develop with normal leaves where internode does not elongate (Figure.8K). Mutants like tall growth pattern with intensified internodes and single branched, bushy plants by increased branching, plant with stunted growth habit, and variation in pod size (Figure.9A-E) have been screened from different doses of mutagens. The recurrence of morphological variants intensified upon rising mutagenic doses influencing nearly all portions of the plants was detected. The mutants were screened on the basis of different groupings, for instance, plant size, plant height, leaf morphology, pod shape, and size. The disparity in the recurrence of morphological variation ascribed to a number of genes through pleiotropic effect (Filippetti and De Pace, 1986) [20]. Plant size and pattern (tall, dwarf, bushy), leaf morphology (size, shape, colour), pod variation (shape and size) were considered monogenic recessive (Shahwar *et al.*, 2019) [55]. Mutant evaluated, induced by the action of MMS, showed enhanced height and a higher number of branches and this may attributed to the deprivation of apical dominance leading towards lateral distribution of growth hormone and consequently enhanced branching. The leaf anomalies may due to mutations that are undoubtedly produced in leguminous plants or else ascribed to chromosomal aberrations (Blixt, 1972 [13]; Azad, 2014) [9].

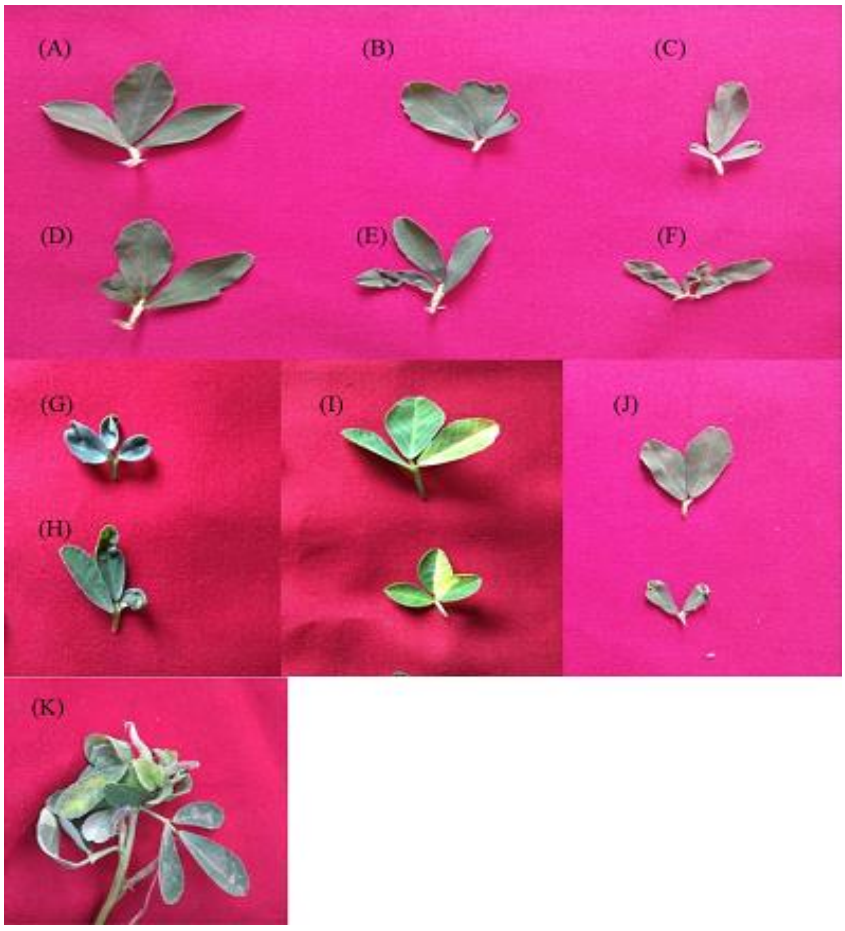


Fig 8: Secluded morphological and chlorophyll discrepancies of leaf in mutated populations; A. Control trifoliate leaflets, B. Unequal leaflets, C. One larger and two smaller leaflets, D. One bilobed and other standard leaflets, E. One deformed and two lanceolate leaflets, F. All three underdeveloped and deformed leaflets, G. Multilobed leaflets, H. Two elliptical with one reduced leaflet, I. Light green and partial chlorotic leaflets, J. Bifoliate leaflets, K. Regular leaves with compressed internode.

Fig 9: Plant height and growth pattern deviations in the mutated population; A. Control plant showing normal growth, B. Plant along with thither branching and prolonged internodes, C. Plant with tall growth habit and single branched, D. Plant with dwarf growth pattern, E. Variation in pod size.

Quantitative Traits

The plant height of mature plants usually decreases with the increasing doses of mutagen in both varieties. The usual height decreased as of 81.21cm (control) to 76.09 cm (0.04% MMS) in var. Desi Methi, despite the fact it reduced from 90.50cm (control) to 86.43 cm (0.04% MMS) in var. Metha (Figure.10A). Plant height decreased significantly with enhancing the level of mutagen population of both the cultivars. The extreme decline in height was observed in 0.04% MMS in both, var. Desi methi and Metha. Similar observations were considered by several researchers (Krishna *et al.*, 1984 [33]; Ansari and Siddiqui, 1996) [6]. Arumugam *et al.* (1997) [8] imply that a reduction in height caused due to the structural variations in the nature of chromosomes or cytogenetic deterioration are key factors for growth arrest. However, growth arrest may also appear from the disturbance of mutagens along with the cell expansion (Sparrow and Sparrow, 1965) [62]. Auxin devaluation, physiological disturbances play a significant role in reduction of plant height (Shahwar *et al.*, 2020) [56]. The tall, dwarf and bushy forms, as observed in the current study have been suggested in lentil (Solanki and Sharma, 2003) [61] after mutagenic treatments. Number of fertile branches increased and decreased on/or after controls in all mutagen treatments.

The least number of branches were detected in 0.04% MMS (i.e., 3.88) in var. Desi Methi and 0.03% and 0.04% MMS (i.e., 1.33) in Metha respectively (Figure.10B). The number of pods was found to decreased from 25.08 (control) to 21.96 (0.01% MMS) and 10.60 (control) to 9.80 (0.04% MMS) with the increasing concentration of MMS in var. Desi methi and Metha, respectively. The remarkable positive change in mean values of pods/plant was seen in a lower concentration of MMS in each of the variety (Figure.10C). The average seed setting was higher in Desi Methi than Metha. The maximum number of seeds per pods were recorded in control is 12.33 and 10.46 and decreases to 10.33 and 8.16 (0.04% MMS) in Desi Methi and Metha respectively (Figure.10D). The standard weight of 1000 seeds appearing in controls of each variety specifically Desi Methi and Metha, was 7.25 g and 12.29 g, respectively. The 0.04% MMS concentration showed decreased 1000 seed weight in both varieties (Figure.10E). The average seed yield of Desi Methi and Metha was 2.12 g and 1.25 g in control. While minimum yield was observed 1.89g in 0.04% MMS and 0.95g in 0.04% MMS in Desi Methi and Metha, respectively (Figure.10F). Quantitative characters like pods carrying branches, pods/plant, seeds/pod, 1000 seed weight (g) as well as total yield per plant (g) exhibited a remarkable rise in the mean values upon treatment with the lower doses of the mutagens, whereas at an enhanced level of mutagens showed inhibitory effect.

The reduction in quantitative traits at higher doses of MMS was recorded. The same observation was noticed by Vandana and Dubey (1998) [71] in Vicia faba treated with EMS and DES. Total yield/plant had positive correlation among the number of pods/plant, as the number of pods/plant intensified, a total number of seeds become more intense then ultimately the yield too increased. In mutation breeding, yield is a very significant parameter, which eventually the plant breeder intends to enhance the yield, including other yield attributing characters. Panigrahi *et al.* (2015) [46].

Proposed that amelioration of yield at substantial levels, as well as quantitative deviations, may exhibit balanced gene mutations in the future generations. It was determined that the reduction in quantitative attributes had been ascribed to the chromosomal damage or physiological disruption lead to the plant cells by the mutagen (Thilagavathi and Mullainathan, 2011) [67].

Concisely, the present investigation has exhibited that lower and moderate concentrations of MMS proved to be adequate in enhancing the genetic base for yield-oriented selection in *Trigonella foenum-graecum* and isolating such lines displaying desired shift in mean values, possibly will be estimated in the future generations for favourable performance

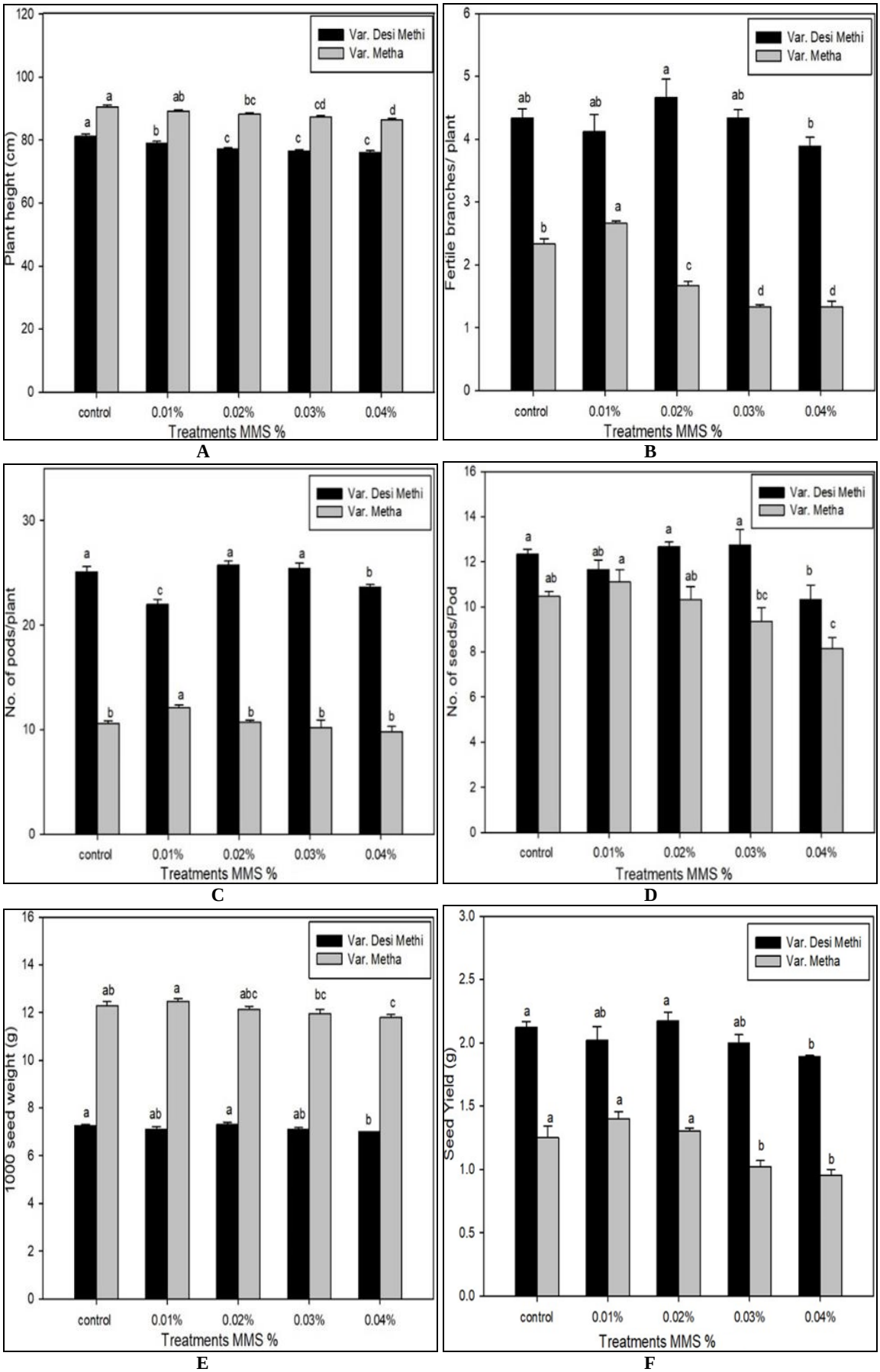


Fig 10: Impact of MMS upon various quantitative traits in M_1 generation of *Trigonellafoenum-graecum* L. (A). Plant height (cm), (B). A number of fertile branches/plant, (C). A number of pods/plant (D). Number of seeds/pod, (E). 1000 seed weight (g) and (F). Seed yield (g). The data represent the mean ± SE; therefore, mean values by the same letters are not distinct at a. 5% significance level (1)=0.05) based on the Duncan multiple range test (DMRT).

Conclusion

Genetic discrepancy in medicinal crops is very limited, hence induced mutagenesis exists as the most significant perspective to broaden the genetic variation and contribute to ease the prospects of plant genetic resources and thereby generate the envisaged designer crop varieties. It was revealed that the extent of the introduction of genetic variability in each of the variety of fenugreek relies on the mutagen doses. The genotype of *Trigonella foenum graecum*, a medicinal plant, can be ameliorated through lower or moderate concentration of mutagen, as it showed an effective shift in the mean values. The findings of the present analysis will assist the researcher in exploring the critical areas of mutation breeding programs to gain the maximum desirable mutations which can be exploited in future generations.

Acknowledgment

We acknowledge the Chairperson of the Department of Botany, Aligarh Muslim University, Aligarh, for providing required amenities and encouraging during the evaluation.

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