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Effect of EMS on chlorophyll mutagen in fenugreek (*Trigonella foenum-graecum* L.)

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Abstract
The present study was conducted to find out the mutagenic effect of EMS [Ethyl Methane Sulfonate (CH_3 OSO_2 C_2H_5)] in the local variety of fenugreek (*Trigonella foenum-graecum* L.). Fenugreek seeds were treated with different treatment of chemical mutagen. Two sets containing 400 healthy seeds were selected for treatment. To determine the LD50 value, fenugreek seeds were presoaked in double distilled water for 6 hours followed by EMS 0.1%, 0.2%, 0.3%, 0.4%, 0.5%, 0.6%, 0.7%, 0.8%, 0.9% and 1.0% concentrations respectively. The main objectives of this study were, to determine the optimum dose of LD_{50} value of EMS in Fenugreek, to isolate chlorophyll mutants based on changes in phenotypic traits.

Keywords: *Trigonella foenum-graecum*, mutagens, EMS, lethality

Introduction
Trigonella foenum-graecum L. (2n=16) commonly known as fenugreek (methi), an annual dicotyledonous herbaceous plant belongs to the family Leguminosae with branched stems, trifoliate ovate-orbicular leaves, roots bearing nodules, white flowers, papilionaceous corolla, stamens diadelphous [1+(9)], ovary superior, ovules many, pods bearing golden yellow seeds. Seeds vary from rectangular to round in outline with a deep groove between the radical and cotyledons. The young plants serve as vegetable for human consumption seeds as a spice or as herbal medicine (Petropoulos, 2002) [1]. Fenugreek leaves and seeds have been used extensively to extracts and powders for medicinal uses (Basch *et al.*, 2003) [3]. Fenugreek is reported to have anti-diabetic, anti-fertility, anti-cancer, antimicrobial, anti-parasitic and hypocholesterolemic effects (Al-Habori and Raman, 2002) [3]
In India, Fenugreek is used as a stimulant (Tiran, 2003) [4]. Fenugreek seed in powdered or germinated form exhibits anti-diabetic properties (Broca *et al*., 2004) [5] hypocholesterolemic effects, anti-cancer effects and effect on thyroxine induced hyperglycemia. Other pharmacological properties of Fenugreek include anti-inflammatory, antiulcer, sexual stimulant and antioxidant. The biological and pharmacological properties of fenugreek are attributed to the variety of its constituents, namely; steroids, N -compounds, polyphenolic substances, volatile constituents, amino acids etc. (Sharvan and Richa, 2014) [6].
Fenugreek seed contains 45-60% carbohydrates, mainly fibre (galactomannans), 20-30% proteins high in lysine and Tryptophan, 5-10% fixed oils (lipids), Pyridine alkaloids, mainly trigonelline (0.2 0.38%), choline (0.5%), free amino acids, such as 4-hydroxyisoleucine (0.09%), arginine, histidine and lysine, calcium and iron, saponins (0.6 1.7%), glycosides yielding steroidal sapogenins on hydrolysis (diosgenin, yamogenin), cholesterol sitosterol, vitamin A, B, C and nicotinic acid (Budavari, 1996) [7]. Fenugreek is an important cultivated crop in parts of Europe, Northern Africa, West and South America and Australia (Acharya *et al*., 2006) [8]. Major fenugreek producing countries are Russia, India, Pakistan, Germany, Argentina, Egypt, Canada, Iran, Canada, USA and China (Basu, 2008) [9]. India is the largest producer of fenugreek in the world where Rajasthan, Gujarat, Uttaranchal, Uttar Pradesh, Madhya Pradesh, Maharashtra, Haryana and Punjab are the major fenugreek producing states (Debranjan and Tara, 2010) [10]. Rajasthan produces the lion's share of India's production, accounting for over 80% of the nation's total fenugreek output.

Mutagens
Plant breeding is often regarded as an important one of the branches among applied genetics. It forms the most important breakthroughs in the history of genetics that led to the discovery of

experimental mutagenesis in the early 21st century. Mutation techniques can generate genetic variation and increase the desired characters significantly in plants of new cultivars. The application of mutagenesis in agriculture for improving the crop plants presented a new departure from the conventional breeding methods. In conventional breeding methods, the store of natural variability present either in the base population initially or introduced through hybridization, is subjected to recombination and selection so as to increase the frequency of favourable combinations of genes in the selected line.

Mutation breeding helps in greater magnitude of variability in various plant traits in a comparatively shorter time. Mutation is a sudden heritable change in an organism and generally a structural change in genes. Mutation produced by changes in the base sequences of genes (as a result of base pair transition or transversion, deletion, duplication or inversion etc.) are known as gene or point mutations. Some mutations produced by change in chromosome structure, or even in chromosome number are known as chromosomal mutations. The induced mutations are caused artificially by mutagenic factors. The agents that induce mutations are called mutagens and mutagens mainly consist of two different kinds; radiation (physical) and certain chemical mutagens. Mutagens are not only beneficial to create genetic variability in a crop species, but also useful for the effective control of pests during post-harvest storage (Chaudhuri 2002) [11].

Practicing of induced mutation for crop improvement is known as mutation breeding. Mutation breeding has been widely used for the improvement of plant characters in various crops. It is a powerful and effective tool in the hands of plant breeders especially for autogamous crops having narrow genetic base. Mutation induction offers significant increase in crop production and the possibility of inducing desired attributes that either cannot be found in nature or have been lost during evaluation. Treatments with mutagens alter genes or break the chromosomes. Gene mutations occur naturally as errors in DNA replication. Most of these errors are repaired but some may pass on to the next cell division to become established in the plant offspring as spontaneous mutations. Gene mutations without phenotypic expressions are usually not recognized. Consequently, genetic variation appears rather limited and breeders have to resort to mutation induction (Adamu and Aliyu, 2007) [12]. Mutagenic agents have been used to induce useful phenotypic variations in plants for more than seventy decades (Anitha Vasline *et al*., 2005) [13].

Ethyl methane sulphonate (EMS)

Ethyl methane sulphonate (EMS) is a chemical mutagen of the alkylating group and has been commonly used in plant breeding because it can cause high frequency of gene mutations and low frequency of chromosome aberration. EMS alkylates guanine residues, producing O6-ethyl guanine, which pairs with T but not with C (Chandra Mohan *et al.*, 2016) [14]. As a result, replication of un-repaired alkylation damage will effectively replace the G/C base pair with an A/T. This mechanism predicts a strong G/C to A/T bian in EMS induced mutations, as observed in numerous mutagenic studies. In fenugreek, several have tried for artificial induction of mutations through the use of mutagens (Basu *et al.*, 2008) [9]. Despite the release of different cultivars, fenugreek production has not increased to any noticeable extent over the last decades. The present work is therefore, designed to evaluate the morphological and cytological effects of chemical mutagens in fenugreek with the main objective of inducing changes in the genotype to enhance genetic variability in this plant as to broaden its genetic base for selection of desirable genotypes for commercial cultivation (Chandra Mohan *et al.*,2016) [14].

Materials and Methods

The dry and dormant seeds of the fenugreek (*Trigonella foenum-graecum*) local variety were treated with EMS treatments were used in the present study. The present study was carried out in Pachaiyappa College Botanical Garden.

Mutagen Used

The seeds of fenugreek were treated with different treatment of chemical mutagen. The chemical mutagens used were Ethyl Methane Sulfonate [EMS (CH_3 OSO_2 C_2H_5)]. The chemical was obtained from HI-MEDIA laboratories, Mumbai, having a half-life period of 30 hours with a molecular weight of 124.16 and density of 1.20.

Two sets containing 400 healthy seeds were selected for treatment. To determine the LD50 value, fenugreek seeds were presoaked in double distilled water for 6 hours followed by EMS 0.1%, 0.2%, 0.3%, 0.4%, 0.5%, 0.6%, 0.7%, 0.8%, 0.9% and 1.0% concentrations freshly prepared solution for 3 hours. After the EMS treatment, the treated seeds were washed thoroughly in running tap water to terminate the residual effect of the mutagenic chemicals. After the completion of the treatment the treated seeds were sown immediately in the field along with their respective controls to raise the M_1 generation in a randomized block design with three replications. The seedling height reduction (I) in different M1 generation was studied following Nilan *et al.*, (1965) [15] and Velu *et al.*, (2007) [16].

The plant survival (L) was computed as the percentage of plants surviving till maturity. The biological damage (lethality/injury) was computed as the reduction in plant survival and plant height. The respective control and treatment progenies were screened several times for morphological mutations throughout the crop duration. Different kinds of chlorophyll mutants (Albino, Xantha, Chlorina, and Viridis) were scored from emergence till the age of four week in M1 generation by using modified classification of Kharkwal (1998) [17]. Mutation frequency was calculated as percentage of mutated M1 progenies for both chlorophyll and morphological mutations in each treatment. The Mutagenic effectiveness and efficiency were calculated on the basis of formulae suggested by Konzak *et al*., (1965) [18].

- Mutagenic effectiveness (Chemical mutagens) = Mf×100/c×t.
- Mutation frequency = Chlorophyll/Viable mutants per100 M1 plants.
- C=Concentration of mutagen in mM.
- t=Period of treatment with chemical mutagen in hours.
- L=Percentage of lethality (or) survival reduction.
- I=Percentage of injury (or) reduction in seedling size.

Chlorophyll Mutants

The chlorophyll mutants were scored on 20th day after sowing. Four types of chlorophyll mutants were observed in treated plants.

Albino: Albino-white, lethal, no chlorophyll (or) carotenoids are formed.

Xantha: Carotenoid pigment predominantly found but chlorophylls are not formed.

Chlorina: Light green to flush yellow green, mostly viable seedlings. They change to normal condition.

Viridis: Light yellow patches, along with green following either regular or irregular pattern and viable.

Results

The present investigation was undertaken in order to study the artificial inducement of mutation in fenugreek local by using EMS mutagens through the biological changes in M_1 generation. This was aimed to find out the economic potentialities of the viable mutant and the nature of induced variability in the qualitative and quantitative traits in M1 generation.

M_1 Generation

LD_{50} for Gamma rays and EMS

Data on the effect of mutagens on germination, expressed as per cent control and LD_{50} is presented in Table 1. The untreated seeds of genotype had 100 per cent germination. The germination percentage decreased with increase in the dose/conc. of the treatment. Fenugreek seeds were treated with EMS, showed reduction in germination at higher concentration. Lowest germination percentages (2.1%) were observed at 1.0% of EMS on 7th day after the germination. Based on the germination studies, 50% lethality was observed at 0.4% of EMS (Plate-1), (Table-1)

Plate 1: Seed Germination on 7th day at laboratory condition

Table 1: Determination of LD_{50} value for Ethyl methane sulphonate in fenugreek

EMS treatment (Conc. mM)	Seed Germination (%)	Percent of over control
Control	**95%**	-
0.1%	93%	97.8%
0.2%	72%	75.7%
0.3%	64%	67.3%
0.4%	48%	50.5%
0.5%	42%	44.2%
0.6%	38%	40%
0.7%	25%	26.3%
0.8%	17%	17.8%
0.9%	9%	9.4%
1.0%	2%	2.1%

Observation of Chlorophyll mutants (M_1 generation)

The chlorophyll mutants were scored on 20th day after sowing. Four types of chlorophyll mutants were observed in treated plants (Plate-2 and Table-2).

Albino: Albino-white, lethal, no chlorophyll (or) carotenoids are formed.

Xantha: Carotenoid pigment predominantly found but chlorophylls are not formed.

Chlorina: Light green to flush yellow green, mostly viable seedlings. They change to normal condition.

Viridis: Light yellow patches, along with green following either regular or irregular pattern and viable.

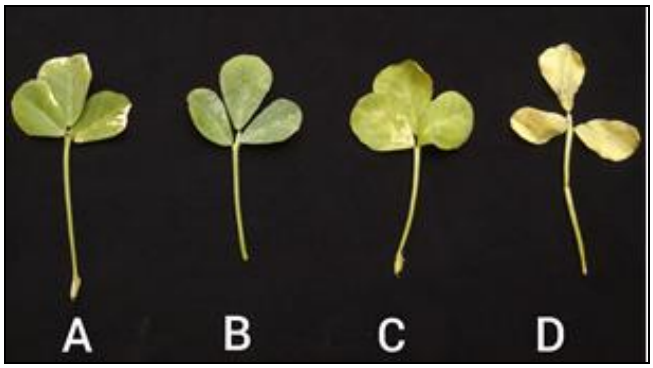

Plate 2: A) Albino, **B)** Xantha, **C)** Viridis, **D)** Chlorina

Table 2: Effect of EMS on frequency of chlorophyll mutants in M1 generation of fenugreek

EMS treatment	Total no of plants studied	Spectrum of Chlorophyll mutants				Total number of Chlorophyll mutants	% of mutants frequency
		Albino	Xantha	Viridis	Chlorina		
0.1%	15	-	-	-	-	0	0
0.2%	13	-	-	-	-	0	0
0.3%	12	-	-	1	-	1	8.33

0.4%	9	1`	-	1	1	3	33.3
0.5%	7	1	-	1	-	2	28.57
0.6%	5	1	-	-	-	1	20.1
0.7%	2	-	-	-	-	0	0

M_1 generation (Laboratory Studies)

LD_{50} Value

The availability of efficient seed germination system after the mutagenic treatment is crucial in achieving successful mutagenesis. The higher exposure of gamma rays may cause injury in seeds and usually show inhibitory effects on seeds of Angiosperms and Gymnosperms. Compared to physical mutagen, the germination of seeds reduced more under chemical mutagen that they damage the biological material as reflected in the quantitative parameters (Gaul, 1964). In the present study to find out optimum dose/conc. of the mutagens, germination percentage of the seeds was calculated with effect of chemical mutagens of fenugreek. Among the mutagenic doses/conc., of the LD_{50} (optimum) value was recorded at 0.5% of EMS (51.43%, 51.05%) and the maximum reduction of germination percentage were noted at 0.8% of EMS showed more lethal effect of fenugreek (Table 1). Similar results were noted in sesame (Mensah *et al*., 2007) [21] and *Lepidium sativum* (Majeed and Muhammad, 2010) [22].

Chlorophyll mutation (Field work)

Leaf color mutations are one kind of most frequently observed mutation in both spontaneous and induced mutant populations, and often used as an indicator of mutagenic effects and efficiency of various mutagens**.** Chlorophyll development seems to be controlled by many genes located on several chromosomes, which could be adjacent to centromere and proximal segment of chromosomes (Swaminathan, 1964) [23].

In the present investigation, different chlorotic abnormalities were scored in fenugreek plants. The chlorophyll mutants were observed in different doses/concentrations of gamma rays. They were albino, chlorina, viridis and xantha. 0.6% of EMS was higher frequency of chlorophyll mutations local fenugreek varieties. The green-revertible albino mutation was observed in 0.6% of EMS regarded as a better mutant because it seldom affects the growth of such lines at late stage, and is more visibly different from green ones when compared with chlorina mutants. The seedlings appeared white were much dependent on the growth temperature, the higher temperature shorter the time it took for the leaves to turn into green in rice (Shen *et al*., 2004) [24].

Chlorina mutation was observed in 0.6% of EMS respectively. For the chlorina mutation, as there were colour level differences among both mutant and normal seedlings. It became particularly worse when seedling growth was subjected to environmental stress, such as low nitrogen level and extremely low/high temperatures (Zhou *et al*., 2006) [25]. The xantha mutations were noted in 0.6% of EMS of fenugreek. It is expressed uniformly in all tissues of the plant, e.g., leaves and sheath, during whole growth duration, which made it readily distinguishable from green seedlings. The mutation apparently affected the content of all pigments chl a, chl b, and carotene content at different levels (Zhou *et al*., 2006) [25]. Viridis mutation seedlings are uniform light yellow green color leaves it's a viable mutant observed at 0.6% of EMS of fenugreek varieties. The viridis types were predominant than albino, xantha and chlorina types, irrespective of the cultivar in rice as reported by Prakash and Khanure (2000) [26]. The appearance of greater number of viridis after xantha may be attributed to involvement of polygenes in chlorophyll formation (Ahmad and Cashmore, 1996) [27]. These types of mutations were observed in mungbean (Singh and Singh, 1989) [28], chickpea (Kharkwal, 1999) [29] and in grasspea (Das and Kundagrami, 2000) [30].

Mutation frequency

The mutation frequency was calculated on the M_1 plant basis showed a dose dependable measure of genetic effects in mutagens (Gautam *et al*., 1998). In the present study the spectrum of morphological mutations in two varieties induced included for chlorophyll mutants (albino, chlorina, viridis, xantha)

Summary and Conclusion

In order to find out optimum dose/concentration of the mutagen, germination percentage was calculated with local variety of fenugreek. Based on the seed germination, 50% lethality (LD_{50}) was determined in EMS (0.4%). The chlorophyll mutants were recorded in M_1 generation. Four types of chlorophyll mutants were observed *viz*., albino, xantha, chlorina and viridis. The frequency of mutation was high in 0.7% of EMS.

The results of the experiment indicated that increasing doses of EMS caused severe effects on plant development. In general, according to the results of the present work, the best treatment was the application of 0.4% of EMS dose was stimulate plant growth. The crucial aim of a mutagenic treatment is to induce mutations leading to genetic improvement of a specific trait and selection of economically important mutants. For breeding purposes mutagenic treatments with strong genetic effects are desirable and low physiological effects.

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