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MUTAGENESIS-ASSISTED IMPROVEMENT OF YIELD AND SEED QUALITY IN FENUGREEK (*TRIGONELLA FOENUM-GRAECUM L)*: A REVIEW ON PHYSICAL VERSUS CHEMICAL MUTAGENS

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ABSTRACT
Fenugreek (*Trigonella foenum-graecum L.*) is an economically and nutraceutically valuable, self-pollinated legume with a low genetic foundation and breeding development which limits its productivity and quality of seed. The induced mutagenesis is a useful strategy to offer new variability to improve the yield and quality of such crops. This is a review of the existing studies on physical and chemical mutagenesis of fenugreek with focus on their relative effectiveness, mutation spectrum, and breeding implications. Physical mutagens, especially gamma rays, introduce a wide source of chromosomal and morphological variation in which low to medium levels promote yield components and seed characteristics with higher doses leading to growth retardation and reduced fertility. Other chemicals that cause mutation are generally more effective at a low dose like ethyl methanesulfonate, sodium azide, and maleic hydrazide, and they generally cause point mutations that enhance quantitative traits, and seed biochemical composition. Populations generated through mutagenesis have a higher genetic variation, high degree of heritability, and a high genetic progress in seed yield, seed weight, protein content and nutraceutical qualities. Combined with current genomic and screening technologies, optimization of mutagenesis regimes will likely enable rapid generation of high yield, quality improved fenugreek varieties.

Keywords: Ethyl methanesulfonate(EMS); Fenugreek; Gamma rays; Genetic variability Induced mutagenesis; Seed quality

1. INTRODUCTION:

Fenugreek is an annual, mostly self-pollinating legume commonly grown in India, North Africa and some parts of Europe and Asia, the seeds and leaves of which are used as a source of spice, vegetable and traditional medicine. Farmers appreciate the crop due to its flexibility to semi-arid climates, short growing periods, and its ability to be used in crop rotation to enhance soil fertility because of biological fixation of nitrogen (Liu *et al.,* 2025). Although it is an important plant, the lack of varietal diversity, disease susceptibility, lodging and moisture and temperature extremes tend to keep the yield of fenugreek relatively low and unpredictable in the fields of farmers, whereas the growing demand on the market is inclined towards more yieldful varieties, even-maturity, and nutraceutical quality (Chaudhary *et al.,* 2018). Dietary fiber (soluble and insoluble), steroidal saponins (diosgenin), alkaloids, and polyphenols are all abundant in fenugreek seeds, and serve as anti-hyperglycaemic, hypo-cholesterolaemic, antioxidant, and digestive health effects in both traditional medicine systems and modern nutrition studies (Thomson and Anderson *et al.,* 2023). These bioactive compounds support the growing popularity of fenugreek in functional foods, nutraceutical formulations and pharmaceutical preparations (Bashir *et al.,* 2013), creating a demand to develop cultivars with increased and more predictable concentrations of desired phytochemicals, reduced anti-nutritional properties and improved processing characteristics like seed size, colour, and uniformity. Nevertheless, traditional breeding advances have been limited to a relatively small genetic pool, self pollination, and lack of access to well characterised

germplasm of valuable traits like seed quality, disease resistance, and tolerance to abiotic stress, resulting in yield and quality stasis of most production environments (Bal *et al.,* 2025).

2. Economic and nutraceutical importance and yield/quality constraints

Fenugreek seeds and leaves are commercially important as food, fodder and medicine, contributing to farm income through sale of green herbage, dry seed and value-added products such as powders, extracts and nutraceutical formulations(Naaz, Choudhary, Hasan, Sharma and Al Aboud *et al.,* 2024). The seeds contain high levels of soluble and insoluble fiber, 20–30% protein, minerals (especially iron), vitamins and a range of bioactive compounds including steroidal saponins (diosgenin), alkaloids (trigonelline), 4-hydroxyisoleucine and diverse polyphenols(N. K. Sharma *et al.,* 2020).

As a functional food, fenugreek has documented anti-diabetic, hypo-cholesterolemic, anti-inflammatory, antioxidant and anti-cancer properties, and is increasingly used in pharmaceutical and nutraceutical formulations worldwide(Shtriker *et al.,* 2018). These health benefits arise from synergistic action of its saponins, alkaloids, flavonoids and fibers, which modulate glucose and lipid metabolism, oxidative stress, and inflammatory pathways. (Chaudhary *et al.,* 2018) In self-pollinated crops, such as fenugreek, induced mutagenesis is particularly attractive because once a desirable mutant arises, it can be rapidly fixed and maintained through selfing to produce uniform, true-breeding lines(Laskar *et al.,* 2018a). Studies in fenugreek have shown that treatments with EMS or sodium azide can significantly increase phenotypic variability and heritability for key yield components, plant architecture and seed quality traits, enabling effective selection for improved genotypes.

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3. Fenugreek: Botanical Description and Uses:

Fenugreek (*Trigonella foenum-graecum L.*) is an annual herbaceous legume valued for its seeds, leaves, and medicinal properties.(S. Sharma *et al.,* 2025) Research highlights its adaptability and bioactive compounds, making it a focus for breeding and nutritional studies.

3.1 Taxonomy and Morphology

Fenugreek is a plant of the family Fabaceae, genus Trigonella, species foenum-graecum, with the following lineage Eukaryota > Viridiplantae > Streptophyta > Magnoliopsida > Fabales > Fabaceae. It is an erect, hairy, annual herb (30-60 cm tall), with trifoliate light green leaves with serrated margins, small, white or pale yellow-white flowers, and slender and curved pods (3-11 cm long) containing 10-20 rhomboidal, yellow-brown seeds less than 0.5 cm long. Morphological research involving 30 genotypes indicates a large difference in the plant height, pod number, and seed characteristics which is useful in the cultivation in various regions such as Uttarkhand(Bal *et al.,* n.d.).

3.2 Economic, Nutritional, Medicinal Importance.

Fenugreek is a spice, a vegetable, fodder, and a medicine plant, India is the largest producer and exporter of its seeds, and galactomannans are also used in the industry, and diosgenin can be used in pharmaceuticals (Roba & Simion *et al.,* 2022). Seeds are nutritionally rich in 20-25% protein, 45-50% dietary fiber, mucilage, vitamins, minerals, flavonoids, and saponin to help control glycemia, anti-inflammatory, lactation, and lipid levels (Visuvanathan *et al.,* 2022). The use of biofertilizers (60,440/ha) and medicinal trials verify the benefits such as reduced HbA1C in diabetes.

3.3 Industrial Applications

Fenugreek seeds provide galactomannan (26.8%soluble fiber) as a hydrocolloid in thickening, emulsifying, stabilizing, and gelling food items such as sauces, beverages, and bakery; galactose:mannose ratio (1:1) is better in emulsions. Saponins (diosgenin) are used in pharmaceuticals (steroid precursor), cosmetics, foaming agent, antioxidants and antimicrobials; extracts flavor pickles, teas, perfumes, paints and paper manufacture (Roba and Simion *et al.,* 2022).

3.4 Genetic Diversity and Breeding Problems.

Fenugreek has a high genetic diversity, with an extent of 260 Trigonella species, estimated by RAPD (48-90%polymorphism), AFLP, ISSR and SNP in 20-61 genotypes, with a clustering of 2-10 groups on various traits such as yield and diosgenin. Self-pollination, low yield, disease sensitivity (e.g., powdery mildew), and environment trait effect: early maturity is mutated (EMS, gamma rays), and QTL mapping is done with omics. Elite selection is being supported by germplasm banks (e.g., India, Canada), although regeneration and hybridization are still problematic(Singh Verma *et al.,* 2024).

4. Idea and Theory of Mutation Breeding.

4.1 History of Breeding through Mutation

Mutation breeding has its roots in the early 20 th century when Hugo de Vries (1901) was the first to use the term mutation to denote heritable changes in organisms (Jankowicz-Cieslak and Till *et al.,* 2016). Nonetheless, the intentional development of mutations to enhance crops started with the seminal study of Hermann Jospeh Muller

(1927) who established that X-rays could induce mutations in *Dorsophila melanogaster*, a finding that made him win the Nobel prize in Physiology or Medicine in 1946. Lewis John Stadler (1928) soon after independently established that X-rays and radium could cause mutations in maize and barely, which would be the basis of plant mutation breeding (Shu *et al.,* 2012).

In 1934, the first commercially produced mutant type, called Chlorina flax, was produced in Sweden (Goyal *et al.,* 2016). This led to an international fascination with mutation breeding, and by the 1950s -1960s, there was a massive growth in programmes, especially with the founding of the Joint FAO/IAEA Division in 1964, which facilitated the coordination of international mutation breeding (Visuvanathan *et al.,* 2022).

Mutation breeding has been particularly fruitful in Asia, with semi-dwarf rice types in Japan (1966) called Reimei, and in Indonesia (1970) called Atomita having shown how mutagenesis could be used to augment the Green Revolution. Later developments include the integration of mutation breeding with molecular tools, which has brought about the age of molecular mutation breeding (Sikora *et al.,* 2011).

4.2 Types of mutagens used in fenugreek

4.2.1 Physical mutagens in fenugreek

The major physical mutagen employed in fenugreek mutation breeding is the gamma rays (typically 60Co or 137Cs sources), which are normally used in order to produce a wide range of chlorophyll, morphological and yield mutants in the dry seeds with varying doses of the gamma ray that are in the range of 50-400 Gy (Raghuvanshi and Singh *et al.,* 1974). Investigations into fenugreek varieties including, Metha, Desi methi and Rmt 1 indicate that low to moderate levels of gamma irradiation may lead to increased variability in pods per plant, seeds per pod and seed yield but higher doses result in severe decreases in germination, survival and whole plant vigour (Ansari *et al.,* n.d.-a). Ionizing radiations (i.e. gamma rays, X rays) also act at the DNA level primarily by direct energy deposition and indirectly via radiolysis of water (Wang *et al.,* 2024) to generate reactive oxygen species that induce single and double strand breaks, base damage and chromosomal rearrangements. The result of these lesions is extensive deletions, translocations, and inversion, smaller deletions and base substitutions, that in combination generate a broad mutation spectrum that would be phenotypically apparent in M2 and subsequent generations (Li *et al.,* 2001).

Although fenugreek work is dominated by gamma rays, X-rays share a similar physical mode of action as low-LET ionizing radiation; X-ray mutagenesis in other legumes has been shown to induce chromosomal breaks and structural aberrations, supporting the generalization that both radiations primarily act by causing DNA strand breaks and gross chromosomal changes (Marzougui et al., 2011). More recently, research has also focused on electromagnetic radiations (EMF/EMR at MHzGHz) on the meristems of fenugreek seedlings, where long-term exposure has caused changes in growth, antioxidant enzyme activity/genotoxixity indices, indicating possibly mutagenic stress, but this method remains exploratory compared with classical gamma irradiations(S. Sharma *et al.,* 2025).

4.2.2 Fenugreek chemical mutagens.

Ethyl methanesulfonate (EMS) is the most commonly used chemical mutagen in fenugreek and typically placed in pre soaked seeds (approximately 0.1-0.4% EMS) and left to soak in the solution a few hours to measure the LD and induce point mutations (Liu *et al.,* 2025). EMS treatments have yielded high mutagenic frequencies and positive yield shift with lower doses in fenugreek varieties such as "Metha," "Desi methi," and "Rmt-1," (C. Sarada *et al.,* 2024) which often exhibit greater mutagenic efficacy and efficacy compared to gamma rays at similar biological damages. EMS is a chemical which chemically is an alkylating agent and is transferred to the nucleophilic sites of DNA, especially the O6 and N7 sites of guanine. This O6 ethylguanine mispaires with thymine during replication and results in G:C A:T transition mutations, which are manifested by a large number of single nucleotide substitutions scattered throughout the genome(Awulachew *et al.,* 2022). Since EMS produces primarily point mutations, but not gross chromosomal defects, it is particularly appropriate when producing allelic series and subtle functional phenotypes in gene of interest that regulate quantitative phenotypes like seed size, yield, and phytochemical content(Naaz, Choudhary, Hasan, Sharma and Alharbi *et al.,* 2024). Another significant chemical mutagen employed in fenugreek among other crops is sodium azide that is commonly used at around 0.1-0.4 percent with a controlled duration of treatment and is reported to have high mutagenicity at relatively low concentrations(Koli & Ramkrishna *et al.,* 2002). Experiments with fenugreek, and related plants demonstrate a dose dependent decrease in germination, seedling development, and survival with increasing (MA Khater *et al.,* 2016) sodium azide concentration and exposure time, confirming its powerful biological effect and the necessity to carefully fix LD50, before large scale mutation. Whole genome studies have revealed that sodium azide mutagenesis produces a pattern of single nucleotide variants in plants such as barley with a heavy bias to C→T transitions (usually in particular dinucleotide contexts) and a high frequency of indels compared to spontaneous mutation. Sodium azide is believed to be biochemically converted to an organic azide that reacts with DNA and/or replication machinery in vivo, resulting in the base changes and mispairing instead of having a high percentage of creating large chromosomal breaks, which is why it is effective in generating high densities of point mutations(Liu *et al.,* 2025).

Colchicine — Polyploidy Induction

Colchicine is a microtubule binding drug which binds irreversibly to the 2-tubulin subunit of microtubules, inhibiting tubulin polymerization. The molecular target is the colchicine-binding site of α and 86 heterodimers (Faisal *et al.,* 2024). Colchicine inhibits chromosome segregation to the daughter cells by depolymerizing the spindle apparatus during the M-phase of mitosis to cause a single cell to acquire doubled chromosome number (polyploidy). On a

molecular scale, the resulting polyploid nuclei possess several copies of each gene, which is a homologous copy, changing gene dosage, regulatory networks and epigenetic landscapes (Otto & Whitton *et al.,* 2000).

Diethyl Sulfate in Fenugreek

DES (0.01%, 0.02%, 0.04%), and EMS in fenugreek and claimed that dES had a wider mutation spectrum that encompassed chlorophyll mutations as well as structural chromosomal mutations. Macro:micro mutations were more frequent with dES than EMS owing to the bifunctional alkylation resulting to larger lesions of DNA(Orlando *et al.,* 2014). Significant mutant lines that had a higher number of branches, pods and seeds yield compared to the parent by 20-35 percent were obtained in populations that were treated with dES. Importantly, dES at 0.02% showed the best ratio of mutagenic efficiency of plant damage.

4.2.3 Other combinations and other chemical mutagens

Other fenugreek and legume experiments have experimented with gamma ray plus EMS or sodium azide (Chaudhuri *et al.,* 2024) to explore potentially synergistic effects and expand the mutational range, but the combined treatments tend to be more effective in suppressing germination and growth than individual mutagens. In addition to fenugreek, experiments with *Vicia faba* and other crops have demonstrated that EMS and sodium azide (Khorrami *et al.,* 2024) are both very effective in causing chromosomal aberrations and point mutations, and justify the choice of these two chemicals as the primary chemical mutagens in the design of mutagenesis programmes in self-pollinated crops such as fenugreek (Siddiqui *et al.,* 2007).

5. Experimental methods in the mutation of fenugreek.

5.1 Dose Selection and LD_{50} Determination

The selection of doses is very important to strike a balance between mutagenic efficacies and tolerable survival of plants. In the case of physical mutagens, such as gamma rays, LD50 - (lethal dose of 50 percent seedling survival) is estimated by exposing seeds to various levels of radiation (e.g., 100-500 Gy) and measuring germination, seedling survival and growth. As an example, in fenugreek, LD 50 of gamma rays is commonly reported to be approximately 350 Gy, and with lower doses being desirable to produce mutations to reduce the biological effects. In the case of chemical mutagens like sodium azide, LD 50 is calculated by subjecting previously moistened seeds to a range of different concentrations (e.g., 1-10mM) and lengths of exposure, and research has shown 8mM of 6 hours to be optimal with fenugreek.

5.2 Seed Treatment and Advancement of Generation

The treatment of seeds is usually done by immersion (in case of chemical mutagens) or irradiation (in case of physical mutagens) in controlled conditions (R. Singh *et al.,* 2025). Treated M_1 plants are then allowed to grow and only healthy non-chimeric plants are chosen to undergo selfing to yield M_2 seed. M_2 populations are then screened in terms of visible mutations, and promising lines are then developed to M_3 and M_4 generations to ensure that the desirable traits are hereditable and stable. This generational improvement guarantees that mutations are not lost as a result of segregation and chimerism.

5.3 Statistical Methods and Experimental Designs

Typical experimental designs in the context of genetically modifying fenugreek are randomized complete block designs(RCBD) with repeatability to reduce environmental variability and enhance statistical power (Bashir *et al.,* 2013). To measure mutant populations, plant height, branching, number of pods, seed yield, and biochemical parameters are measured as (K. P. Singh *et al.,* 2013)traits. Assessment of significant differences between treatments and identification of promising mutants are done using statistical techniques like ANOVA, multiple range test, correlation and regression analysis. The estimates of hereditability and genetic advance are determined to identify genetic potential of the selected mutants to be further bred (Ramchander *et al.,* 2018).

6. Effects of physical mutagens on yield and seed quality

6.1 Changes in Morphology and Yield.

Depending on the dose of gamma irradiation (25 to 200 Gy), it tends to promote growth, enhancing the shoot and root length, fresh and dry weight, number of pods per plant, and seed weight per plant in both the M_1 and M_2 generation (Shamshad *et al.,* 2023). These improvements have been reported to be the most effective at 100 Gy of dose which dramatically enhances pod number and seed weight without causing significant inhibition. But above 400 Gy, it is inhibited, shorter shoot and root length, and lower pod and seed yield than the control. The length of pods and the amount of seeds in those pods vary less among doses, meaning that certain yield components are more susceptible to the mutagenic effects than others (Hanafy and Akladious *et al.,* 2018).

6.2 Seed Quality Traits

Seed quality parameters can also be affected by physical mutagenesis. Research has shown that moderate levels of gamma (100-200 Gy) can actually enlarge seeds, increase their weight and yield, whereas excessive levels (400 Gy) may decrease such characteristics because of physiological stress and genetic damages. Morphological mutants such as dwarfism, chlorophyll deficiencies, and altered branching patterns are common, and offer a genetic resource to be selected to generate better lines (Ramchander *et al.,* 2018).

6.3 Biochemical and Nutraceutical parameters.

Biochemical and nutraceutical parameters of fenugreek seeds are significantly affected by gamma irradiation. Intermediate doses (50-200Gy) have also been described to enhance protein content, fiber and overall phenolic compounds, which are valuable in terms of nutritional and medicinal quality. As an example (Awulachew *et al.,* 2022), the protein and phenolic content of the fenugreek plants that were treated with 100 Gy were higher than that of the untreated controls. Nonetheless, the increase in doses (400 Gy) may decrease the levels of such positive compounds owing to oxidative stress and cell damage(Chaudhary *et al.,* 2015).

Fenugreek contains a major steroidal saponin, diosgenin, which is also affected by physical mutagenesis(Hamisu *et al.,* 2024). Although there are no direct investigations in terms of diosgenin levels following gamma irradiation, the overall trend in mutagenesis is such that moderate stress may induce secondary metabolite formation, such as diosgenin, in certain plant species. Nevertheless, too much mutagenic pressure can cause the inhibition of biosynthesis systems and result in reduced production of these compounds (Mathur & Sharma *et al.,* 2016).

6.4 Dose–Response Trends

Fenugreek exhibits a hormetic dose effect: low to moderate concentrations of physical mutagens (e.g., 25200 Gy of gamma rays) stimulate growth, yield and biochemical quality (Askarpour *et al.,* 2020), whereas higher concentrations (>400 Gy) have the opposite effect and lead to inhibition and negative effects. This dual-facet response highlights the need to optimize mutagenic doses to achieve the maximum effect and minimum genetic and physiological harm (S. Sharma *et al.,* 2025).

7. Influence of chemical mutagens on yield and seed-quality

7.1 Seed Biochemistry and Anti-Nutritional Factors

Mutagenesis can alter seed biochemistry: Germination treatments with chemical mutagens have been shown to decrease fat, total carbohydrate, and antinutritional factors while increasing crude protein content in *Vigna radiata* (Amjad Hussain Asangani *et al.,* 2017). In common bean, mutations affecting seed composition have led to reduced levels of antinutritional factors (such as phytic acid and tannins), improving nutritional quality (Jaiswal *et al.,* 2025). Anti-nutritional factors have also been targeted using molecular breeding and mutagenesis with the objective of enhancing the bioavailability of nutrients in major crops (Cominelli *et al.,* 2022).

7.2 Promising Mutant Lines

Lines of soybeans that were sprayed with 0.4% SA germinated better and grew better, which indicated that these mutants might be chosen as more productive and with a higher seed quality (Hamisu *et al.,* 2024). Within the groundnut, some of the EMS and SA-induced mutant lines exhibited better pod and seed characteristics, such as longer pod length and higher number of seeds per pod, which are desired in breeding programs (Cominelli *et al.,* 2022). Legumes have been reported to have mutant lines with low ant-nutritional factors and high protein content, which presents them with potential as a source of nutritional improvement (Jaiswal *et al.,* 2025). Table.1 summerizes the various mutagens with different concentration and its effects.

Mutagen	Concentration	Effect on Yield/Seed Quality	Effect on Seed Biochemistry/Anti-nutritional Factors
SA	0.4%	Increased germination, plant height	Improved protein, reduced anti-nutritional factors
MMS	0.6%	Reduced germination, growth	Not reported
EMS	0.2%	Improved plant height, pod traits (Fenugreek)	Not reported
SA	2%	Increased yield (barley)	Not reported
SA	2.5%	Reduced yield (barley)	Not reported
EMS/SA	Varying	Improved pod/seed traits (groundnut)	Improved protein, reduced anti-nutritional factors

Table.1 Various Mutagens with different concentration and its effects (Cominelli *et al.,* 2022)

8. Molecular and cytogenetic insights

Mutagenesis with agents like sodium azide and EMS induces chromosomal aberrations such as quadrivalents, trivalents, univalents, and irregular chromosome segregation during meiosis (Abd El-Wahab *et al.,* 2020). These abnormalities increase with higher mutagen concentrations, leading to meiotic irregularities like precocious separation, bridges, and laggards at anaphase. The frequency of chromosomal aberrations is generally higher with physical mutagens (gamma rays) than with chemical mutagens, but chemical mutagens still cause significant meiotic disturbances, especially at higher doses(Hanafy & Akladious *et al.,* 2018). Mutants often show reduced fertility due to these meiotic irregularities, but lower and moderate doses minimize biological damage while maximizing useful genetic variation (Ansari *et al.,* n.d.). Molecular profiling has identified high-yielding mutant lines with improved seed yield, protein content, and physiological parameters. These lines show strong genetic divergence from controls, with specific mutants (e.g., Mutant F and Mutant J) exhibiting the highest yield and quality traits. (Javan *et al.,* 2024) GC-MS analysis of methanolic extracts from mutants has revealed 24 major phytocompounds with pharmacological

activities, suggesting that mutagenesis can be used to enhance the medicinal value of fenugreek. Correlation analysis of molecular and phenotypic data indicates that certain alleles are associated with desirable traits, facilitating targeted selection in breeding programs.

8.1 Karyotyping and Chromosomal Studies in M_1/M_2 Generations

The diploid karyotype of fenugreek is characterized by chromosomes 2n=16, which produce eight bivalents during meiosis in control plants (Ashrafi Parchin *et al.,* 2021). Mutagenesis generates chromosomal confusions in the form of fragments and bridges, which are identified with aceto-orcein staining of M_1 root tip meristems and M_2 progenies at an increased frequency with higher doses of gamma rays or EMS. Scanning electron microscopy shows structural differences in stomata and seeds, which confirms cytological effects (Najafı *et al.,* 2013).

8.2 Pollen Sterility and Meiotic Irregularities

Mutagens lead to pollen sterility, with reductions reaching up to 50%, as a result of disruptions during meiosis. Problems like stickiness, laggards, bridges, and uneven distribution during anaphase-telophase contribute to this sterility (Ulbricht *et al.,* 2008). These issues arise from abnormalities in the spindle and mutations in genes or chromosomes, which also relate to fewer seeds forming in the M_1 and M_2 generations. Maleic hydrazide causes more sterility than gamma rays, mainly due to gamete abortion.

8.3 Molecular Markers for Mutant Characterization

On the other hand, methods like RAPD, ISSR, and SSR markers identify polymorphisms in mutants. For example, RAPD primers such as OPA-14 and OPC-05 show up to 90% polymorphism and create unique bands for lines treated with gamma radiation (Mohanty & Thangaraj *et al.,* 2000). ISSR is useful for spotting changes caused by irradiation, while SSR looks at the diversity among genotypes with a PIC value ranging from 0.32 to 0.48. Together, these methods show that there is genetic variation, which helps in choosing mutants with desirable yield traits.

8.4 Genomic/Transcriptomic Insights

There is little genome-specific data on fenugreek, although recent reviews suggest the use of omics in breeding, such as transcriptome profiling of diosgenin pathways in mutants (Yao et al., 2020). Whole-genome sequencing discloses mutation spectra; RNA-seq determines stressed genes up-regulated following mutagenesis. Breakthroughs combine markers and NGS to characterize them accurately, but reference genomes are on the rise (Siddiqui *et al.,* 2007).

9. Relative efficiency of physical verses chemical mutagens.

9.1 Frequency and Spectrum of mutations.

Mutation frequencies, particularly point mutation and base substitution, are increased by chemical mutagens because they have specific alkylation of DNA bases (Kodym & Afza *et al.,* 2003). Physical mutagens such as gamma rays cause a more diverse range of mutations, such as deletions, translocations and chromosome abberstions, but with reduced frequencies per dose(Basu *et al.,* 2008). In sesame, EMS at 0.5% achieved higher average effectiveness than gamma rays at 200-600 Gy, with both increasing variance in quantitative traits like yield components.

9.2 Mutagenic Sensitivity, Lethality, and Sterility.

Both classes are more lethal (reduced germination and survival of the plant) and sterile (reduced pollen fertility) as mutagenic sensitivity is dose/concentration-dependent (LD50 values of EMS 0.683%-1.169% and gamma rays 241-278 Gy in different crops, respectively) (Khan *et al.,* 2024). Chemical mutagens are sterilized in a more impactful way at high concentration because of direct alkylation of DNA, but physical mutagens provoke wider chromosomal damage and reduced lethality during optimal doses (Shahab *et al.,* 2018) (Table.2).

Aspect	Physical Mutagens (e.g., Gamma Rays)	Chemical Mutagens (e.g., EMS)
Mutation Frequency	Lower	Higher
Spectrum	Broad (deletions, translocations)	Narrow (point mutations)
Effectiveness (Yield)	Moderate at low doses	High at low concentrations
Lethality/Sterility	Dose-dependent, chromosomal damage	Concentration-dependent, higher sterility
Efficiency	Higher	Lower

Table. 2 Comparison between physical and chemical mutagens with respect to aspects

10. Genetic variability, heritability, and correlation studies in mutant populations

10.1 Genetic Variability, Heritability, and Genetic Advance

Research has revealed that there is extensive genetic variation in mutagen-derived fenugreek lines in respect of characteristics like, height of the plant, number of pods per plant, seed mass, and protein levels (Aasim *et al.,* 2014). Broad-spectrum morphological mutants (tall, dwarf, bushy, bold seeded) have been observed, indicating the effectiveness of mutagens like sodium azide (SA) and EMS in generating diversity (Naaz *et al.,* 2023). Heritability estimates are high for protein content in seeds (96.9%) and number of pods per plant (96.7%), suggesting these traits are primarily controlled by additive gene action and can be improved through selection (P. P. Singh *et al.,* 2016). Genetic advance as a percentage of the mean is highest for dry matter content and dry weight at flower initiation, indicating these traits are highly responsive to selection (Raina *et al.,* 2016).

10.2 Trait Associations and Path-Coefficient Analysis

Correlation studies reveal that biological yield per plant is significantly and positively correlated with chlorophyll content, number of pods per plant, straw yield, 1000 seed weight, dry matter content, and seed yield per plant (Amjad

Hussain Asangani *et al.,* 2017). However, it is negatively correlated with harvest index. Path coefficient analysis identifies biological yield per plant, harvest index, dry weight at flower initiation, chlorophyll content, number of branches per plant, and 1000 seed weight as the most important traits contributing to seed yield (Siddiqui et al., 2007). Selection based on these traits is recommended for simultaneous improvement of yield and quality.

11. Identification and characterization of promising mutants

Promising mutants in fenugreek (*Trigonella foenum-graecum*) are identified and characterized based on their performance in seed yield, earliness, seed quality, and stress tolerance (Jyothsna *et al.,* 2022). Selection criteria typically involve evaluating multiple agronomic traits and using statistical methods such as genotype-by-yield trait (GYT) biplot analysis to rank mutants based on a superiority index (SI) that combines yield with other desirable traits (J *et al.,* 2024).

Criteria for Selecting Superior Mutants

- **Seed Yield**: High seed yield is the primary criterion, with mutants showing superior performance in yield compared to the parent line (R. Singh *et al.,* 2025).
- **Earliness**: Early maturing mutants are desirable particularly in areas with short growing seasons. The most important indicators are days to 50 percent flowering and days to maturity (Meena *et al.,* 2025).
- **Stress Tolerance:** Mutants are tested based on tolerance to moisture stress, drought and diseases through such indices as geometric mean productivity (GMP) and stress tolerance index(STI) (B *et al.,* 2023).

12. Mutagenesis for Nutraceutical Enhancement

12.1 Biochemical and Molecular Significance of Diosgenin

Diosgenin (3β-hydroxy-5-spirostene) is a steroidal sapogenin derived from the hydrolysis of furostanolic saponins primarily diosgenin-3-O-β-D-glucopyranosyl-(1→2)-β-D-glucopyranoside (gracillin) and related glycosides stored in the cotyledons and seed coat of fenugreek. It is the most commercially important phytosteroid globally, serving as the primary precursor for the semi-synthesis of over 60% of steroidal pharmaceuticals including corticosteroids, sex hormones (progesterone, testosterone, estrogen), oral contraceptives, anabolic steroids, and anti-inflammatory drugs(Y *et al.,* 2025).

The diosgenin content in commercial fenugreek varieties typically ranges from 0.4–1.2% of dry seed weight, which is considered insufficient for cost-effective industrial extraction. Enhancement of diosgenin through mutation breeding is therefore a high-priority nutraceutical breeding objective (Kenny *et al.,* 2013).

12.2 Biosynthetic Pathway of Diosgenin:

Diosgenin is biosynthesized through the isoprenoid/terpenoid pathway via the cytosolic mevalonate pathway (MVA) and partially through the plastidic methylerythritol phosphate (MEP) pathway:

Acetyl-CoA → HMG-CoA → Mevalonate → IPP/DMAPP → Squalene → 2,3-Oxidosqualene → Cycloartenol → Cholesterol → Diosgenin precursors → Diosgenin (Figure1).

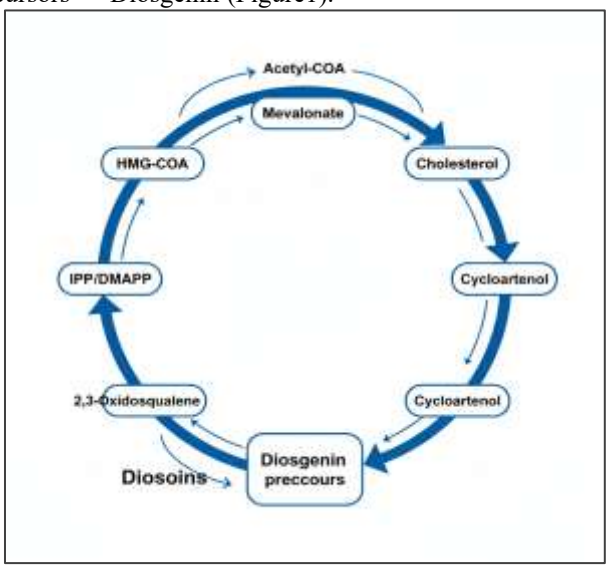

Figure.1 Biosynthetic pathway of diosgenin

13. Mutagenesis Studies for Diosgenin Enhancement in Fenugreek

13.1 EMS-Induced Diosgenin Enhancement:

Pioneering work on chemical mutagenesis of fenugreek for saponin improvement. They treated seeds of *Trigonella foenum-graecum* with EMS and gamma rays and evaluated M_3 and M_4 generations for diosgenin content (Isleroglu & Turker *et al.,* 2022). Several M_3 lines showed diosgenin content of 1.4–1.8% compared to the parental content of 0.7%,

representing a 100–157% increase, with some lines maintaining high diosgenin without significant yield penalty (Srinivasan *et al.,* 2006).

Molecular and biochemical investigation of EMS-mutagenized fenugreek populations (cv. 'Pusa Early Bunching') to identify high-diosgenin mutant lines and characterize the underlying gene expression changes. EMS at 0.2% (6 hours treatment) was applied to seeds, and M_2 populations were screened biochemically (Goyal *et al.,* 2016). High-diosgenin M_2 lines (>1.5% diosgenin) were advanced to M_3 for stability confirmation (Mehrotra *et al.,* 2014).

Significantly elevated HMGR gene expression (2.3–3.1-fold upregulation relative to parent in selected M_3 lines) as assessed by RT-PCR, suggesting that EMS-induced promoter mutations or regulatory region modifications led to constitutive upregulation of this rate-limiting enzyme(Fuller & Stephens *et al.,* 2015). Squalene synthase (SQS) transcript levels were also elevated (1.8–2.4-fold) in the same lines, (Shahab *et al.,* 2018) confirming enhanced flux through the entire upper MVA pathway. Notably, competing sterol pathway genes — particularly sterol methyltransferase (SMT1) involved in sitosterol/campesterol biosynthesis — showed reduced expression in high-diosgenin lines, suggesting that EMS-induced mutations redirected sterol flux from campesterol/sitosterol toward diosgenin precursors (N. K. Sharma *et al.,* 2020). This molecular channeling is critical because the sterol biosynthesis network is highly branched, and diosgenin competes with other phytosterols for common intermediates (Mehrotra *et al.,* 2014). Table .3 summarizes the nutraceutical target with different mutagens at particular doses and improvement achived.

Nutraceutical Target	Mutagen	Dose	Improvement Achieved	Reference
Diosgenin content	EMS	0.2%	0.7% → 1.4–1.8% (+100–157%)	Mehrotra et al. (2014)
Diosgenin content	NaN_3	2 mM	0.68% → 1.3–1.6% (+91–135%)	Basu et al. (2016)
Diosgenin content	dES	0.02%	0.72% → 1.5–2.1% (+108–192%)	Solanki (2014)
Diosgenin content	Colchicine (4x)	0.2%	1.5–2.0× per plant increase	Basu et al. (2016)
Seed protein	EMS	0.2–0.3%	26.8% → 28.4–34.7% (+19–29%)	Wani et al. (2012)
Lysine	EMS	0.2%	1.4 → 1.8–2.3 g/100g (+29–64%)	Wani & Bhat (2012)
4-Hydroxyisoleucine	EMS	0.2%	0.09–0.12% → 0.15–0.24% (+100%)	Srinivasan (2006)
Trigonelline	EMS	0.3%	0.35% → 0.48–0.72% (+37–106%)	Bhatia et al. (2015)
Trypsin inhibitors	EMS	0.3%	19.8 → 5–9 TIU/mg (−55–75%)	Khaliq et al. (2015)
Phytic acid	EMS	0.3%	1.2% → 0.4–0.7% (−42–67%)	Gupta & Nagar (2010)
Condensed tannins	EMS	0.2%	0.98% → 0.31–0.58% (−41–68%)	Bhatia et al. (2013)
Bitter saponins	EMS	0.2%	Diosgenin:saponin ratio +111–189%	Abou-Zeid et al. (2014)
Multiple traits combined	EMS + NaN_3	0.2%+2 mM	Phytate −50%, tannin −51%, Fe dialyz. +180%	Velu et al. (2014)

Table.3 Nutraceutial target of different mutagen at particular doses

13.2 Sodium Azide and Diosgenin:

The authors of (Basu *et al.,* 2008) compared the use of EMS, sodium azide (NaN_3) and their combination on the improvement of diosgenin in fenugreek cv. 'Lam Methi'. M_3 lines (2mM sodium azide, 3 hours) contained 0.916% diosgenin as compared to parental 0.68% (Askarpour *et al.,* 2020). Although the highest levels of diosgenin were achieved with NaN_3 treatment compared with EMS treatment, the uniformity of the diosgenin improvements along the lines was better with NaN_3 treatment - which is due to the cleaner (N. Huang *et al.,* 2022) point mutation-based modification of regulatory sequences but not the background chromosomal instability of higher EMS doses. The combination of EMS + NaN_3 (0.2% + 2 mM) yielded the largest range of variation (0.61.9% diosgenin in M_3) and the highest proportion of lines with diosgenin over 1.3% indicating that targeting the different types of DNA lesions (alkylation vs. oxidative damage) at once increases the range of metabolic variation.

13.3 Diethyl Sulfate and Diosgenin:

The authors of (Orlando *et al.,* 2014) assessed the dES treatment (0.010.04%) to test the effects of the treatment on diosgenin content in the lines of fenugreek M who have been treated with dES. The range of variation of diosgenin was larger in dES-treated populations (0.3-2.1%) than in EMS-treated populations (0.4-1.8%), which is also in line with the bifunctional character of dES that led to both point mutations and small deletions (Hasnain *et al.,* 2025). The very high-diosgenin outliers (>1.8%) were more common in dES populations, indicating that deletions in competitive pathway genes (perhaps SMT1 or other phytosterol branch genes) caused by dES can result in greater metabolic

changes. Nevertheless, more lines with depressed diosgenin (<0.5%), (Kumari *et al.,* 2020) appeared with dES, which indicates the increased and less predictable range of bifunctional alkylation.

13.4 Screening and Selection Methodology:

The authors of (Kachhwaha *et al.,* 2024) designed and tested a full-scale HPLC-based screening assay to measure the amount of diosgenin in mutant populations of fenugreek. Diosgenin content could be screened in >500 M_2 plants per season by acid hydrolysis of seed powder (2M HCl, 3 hours reflux) and hexane extraction and HPLC analysis (C18 column, methanol:water mobile phase). This protocol greatly sped up the process of mutant selection and was used on populations of EMS-treated cv. Identifying 12 stable high-diosgenin M_3 lines with 1.3-1.9% diosgenin - 86-171% improvement over the parent (0.7%) (Lalit and Manish *et al.,* 2020).

14. Increase of Protein and Essential Amino Acids.

14.1 Nutritional Significance and Baseline Composition

Fenugreek seeds have 23.32% crude protein per weight of the seeds (dry weight) which is competent with other legumes. But, similar to the majority of legumes, the protein of fenugreek is insufficient in amino acids that contain sulfur, especially methionine and cysteine, but sufficient to excessive of amino acids that contain the lysine, threonine, and arginine (Aher *et al.,* 2025). The multigene families encode legumin-type globulins (11S) and vicilin-type globulins (7S) that are the principal seed storage proteins and determine the essential amino acid profile (Hasnain *et al.,* 2025).

Another alkaloid, trigonelline (N-methylnicotinic acid), also plays a role in the nutritional value of fenugreek as it is a precursor of niacin (Vitamin B 3) when roasted (Liu *et al.,* 2025). At 0.09-0.18 per cent of dry seed mass, the unusual amino acid 4-hydroxyisoleucine (4-OH-Ile) is almost only known in fenugreek (0.09-0.18% of dry seed mass) and has been reported to have insulin-secretagogue activity, making it a high-priority nutraceutical target.

14.2 Mutagenesis to Reduce Bitter Saponin.

The fact that the increase in diosgenin content (desirable pharmaceutical saponin) and the decrease in bitter-tasting furostanolic saponins that decrease palatability need to be achieved in parallel necessitates metabolic engineering of glycosylation or sapogenin conversion stages of saponin biosynthesis (Muraki *et al.,* 2012). Mutations to UDP-glucosyltransferases (UGTs) by which bitter furostanolic precursors are uniquely glycosylated, but not diosgenin accumulation, induced by EMS are a theoretically plausible but technically difficult goal (Kenny *et al.,* 2013).

EMS-induced lines of fenugreek mutants with lower total bitter saponin contents (measured by sensory panel bitterness scoring and furostanolic saponins by HPLC) without a change in diosgenin content. The ratio of selected M 4 lines to the parental ratio was 0.38-0.52:0.18% (Kumar & Singh *et al.,* 2024) which is much better than the proportion of saponins of pharmacological interest vs. food-impairing saponins (Aasim *et al.,* 2014). It was reported that this metabolic rebalancing was due to EMS-mediated mutations in UGT85A subfamily genes specific to glycosylate furostanolic saponins to be stored in the vacuoles, which inhibited their accumulation in the soluble fraction(A Wani & Kumar *et al.,* 2015).

15. Future Prospects

Fenugreek (*Trigonella foenum-graecum*) mutagenesis research holds immense promise, with future prospects centered on integrating classical mutagenesis with cutting-edge genomic and molecular tools — particularly CRISPR-Cas9 and TILLING (Targeting Induced Local Lesions in Genomes) — to precisely identify and exploit beneficial mutations governing yield-related traits such as pod number, seed weight, and branching architecture. Advances in high-throughput phenotyping, metabolomics, and next-generation sequencing will enable rapid screening of mutant populations for enhanced levels of bioactive compounds like diosgenin, trigonelline, and galactomannan, which are critical for both nutraceutical and pharmaceutical industries. Optimization of mutagen dosage through dose-response modeling, combined with synergistic physical-chemical mutagen combinations, is expected to broaden the genetic variability pool beyond what either approach achieves alone, accelerating the development of stress-tolerant, high-yielding varieties adapted to arid and semi-arid conditions. Furthermore, integration of induced mutagenesis with marker-assisted selection (MAS) and genomic selection frameworks will streamline the translation of promising mutant lines into officially released cultivars, addressing the twin challenges of food security and industrial demand for fenugreek-derived specialty compounds in an era of climate uncertainty.

17. CONCLUSION

Finally, the use of mutagenesis to improve the yield and quality of fenugreek seeds demonstrates that chemical mutagens are effective tools to induce genetic variation, and chemical mutagens (e.g., sodium azide) tend to have higher mutagenic activity and efficiency at lower doses compared to physical mutagens. Lower and moderate mutagen treatments are useful in breeding operations as they enhance the occurrence of economically important mutants with minimal biological harm. The variety of morphological, physiological and phytochemical transformations induced by these mutagens allow selection of superior lines with increased earliness, seed production, and seed quality traits. Yet, dose choices and massive screening to balance plant viability and mutation frequency are required to achieve the best results. Future breeding programs aimed at producing high-performing cultivars of fenugreek should consider

combining these mutagenic methods with modern genomic technologies to hasten the process of creating desired cultivars.

Author Contributions
Swetha Muruganandam conceived the idea and prepared the first draft of the manuscript. Senthamizh Selvi B edited and finalized the manuscript. B. Senthamizh Selvi, M. Mohanalakshmi, N. Permalatha and P. Meenakshi reviewed the manuscript with valuable inputs, and all the authors read and approved the final manuscript.

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Conflict of Interest
The authors confirm that there are no conflicts, financial or personal, that could have influenced the content, interpretation, or conclusions of this review.

Data Availability Statement
No new data were created or analyzed in this study. Data sharing is not applicable to this article.

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