International Journal of Botany Studies
ISSN: 2455-541X; Impact Factor: RJIF 5.12
Received: 12-02-2020; Accepted: 13-03-2020: Published: 14-04-2020
www.botanyjournals.com
Volume 5; Issue 2; 2020; Page No. 74-78

Effects of physical and chemical mutagens on seed germination, seedling height, seedling injury and pollen sterility in m_1 generation of *Clitoria ternatea* L

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Abstract
Clitoria ternatea L. is a well-known herb from fabaceae family which has got phenomenal medicinal properties as an ayurvedic medicine to heal various diseases and used as a legume when tender in many countries. The emperical study on *Clitoria ternatea* L. brought to light the varied antifungal, antioxidant, antidiabetic, hepatoprotective properties which make it a unique medicinal plant. This plant is a rich source of phytochemicals with high level of phenolic compounds. It not only enriches the soil by putrefaction of nitrogen opulent plant material but also acts as a memory booster for human beings. To study the diverse properties of *Clitoria ternatea*. L. under mutation, gamma rays are taken as physical mutagen and Ethyl methanesulphonate as chemical mutagen. This article recapitulates the effects of physical, chemical and combined mutagens on seedling injury, seedling height, germination percentage, and pollen sterility in *Clitoria ternatea* L. in M_1 generation.

Keywords: *Clitoria ternatea* L., ethyl methanesulphonate, gamma rays, mutation, pollen sterility, seed germination

1. Introduction
In spite of numerous methods to cure diseases and their secondary complications, herbal formulation is affordable with minimal side effects. For this reason, the production of medicine as well as dietary supplements derived from plants has been increased in recent years as more than 60% of population opts for them. *Clitoria ternatea* L. is one of the most amply farmed peas because of its exceptional medicinal and nutrient vales all over the world. Mutation induction is key element of plant mutation to enhance the rate of genetic diversity so that breeders can exploit the many varieties in plant breeding programs. An assortment of qualitative as well as quantitative variations has been brought forth by breeding mutations in plants. The development of new improved varieties by generating and utilizing genetic variability through chemical and physical mutagenesis is called as plant mutation. The uninhibited mutation rate is little low and cannot be utilized for breeding. Thus, the plant mutations are propelled by physical and chemical mutagens treatment artificially. Quite many vivid and useful genetic changes have been induced by mutagens treatment such as high yield, change in flower colors, disease free etc., *Clitoria tetnatea* L. plant has wide varieties of secondary metabolites. This plant has profuse pharmacological properties including immunological, cardiovascular, antimicrobial, anti-inflammatory, anticancer, central nervous, antioxidant, anti-inflammatory, anticancer, respiratory, analgesic antipyretic hypolipidemic, and many other pharmacological effects.

1.1 Morphological characters of Clitoria ternatea L
Clitoria ternatea L. is also known as Asian pigeon wing plant has its stems from tropical Asia. This plant is a perennial herb, with elliptic and obtuse leaves. It grows in moist soil as a creeper. The color of flowers is sparkling Deep blue; single, with light yellow markings. The flowers are of 4cm long and 3 cm wider. Some varieties of *Clitoria ternatea* L. yield white flowers. It yields pods about six to ten seeds in each pod of 5-10cm length approximately. They are wholesome when tender and used as a pea and consumed in many parts of the world. The roots of *Clitoria ternatea* L. build a symbiotic association with soil bacteria and fungi and are used to enhance soil quality through the decomposition by making the soil rich in nitrogen.

1.2 Chemical constituents of Clitoria ternatea L.
The major triterpenoids found in *Clitoria tertatea* L. are taraxerol and taraxerone. Anthocyanins, steroids, flavonol glycosides and triterpenoids are some of the secondary metabolites which have been discovered in *Clitoria ternatea* L. The seeds of this plant contain nucleoprotein with its amino acid sequence similar to adenosine, delphinidin3, 3, 5-triglucoside, pentosan, insulin, water soluble mucilage, essential amino-acids. It also contains anti-fungal proteins for plant defensins. Ternatins such as A1, A2,,B1,B2,B3,B4, C1, C2, C3, C5,D1,D2,D3 D3 and preternatins like C4 and A3 are some of the anthocyanins discovered from the tender leaves of *Clitoria ternatea* L.

1.3 Pharmacological effects
We know that many medicinal plants are good alternative sources to find remedies for existing non-communicable diseases now days. The medicinal properties of this plant are scientifically ratified at international level and reported to have specific biological activities.

1.3.1 Antifungal property
A distinctive protein extracted from the seeds of *Clitoria ternatea* L. is a great remedy for a wide spectrum of fungal and bacterial infections (Naz *et al*., 2013).

1.3.2 Antioxidant potential
The phenolic compounds which are abundantly concealed in both the flowers and leaves make *Clitoria ternatea* L. a potential substitute for natural antioxidants (Ligy and Latha, 2013) [5].

1.3.3 Antidiabetic potential
The earlier studies also disclosed that the leaf and flower extracts of *Clitoria ternatea* L. manifested the hypoglycaemic effect. The extracts of *Clitoria ternatea* L. were effective in controlling the biochemical indices of diabetes mellitus (Abhishek *et al*., 2015) [1].

1.3.4 Hepatoprotective potential
Containing *Clitoria ternatea* L. leaves as one of the components, a polyherbal formulation named as "Ayush-Liv.04" showed an antihepatotoxic property against ethanol and CCl4 caused liver damage in rats (Narayanasamy and Selvi, 2005) [13].

1.3.5 Anticancer effect
The life span of an Ehrlich ascites carcinoma tumor bearing mice was increased by decreased tumor volume, viable count and packed cell volume and increased non-viable cell count upon *Clitoria ternatea* L. methanol extracts inoculation (Jacob and Latha, 2012) [5].

1.3.6 Central nervous effect
The seeds and leaves of *Clitoria ternatea* L. are been extensively used as brain tonic and believed as a memory booster and intelligence (Shahnas and Akhila, 2014) [17].

2. Materials and Methods
2.1 Seed collection
Genetically pure seeds of *Clitoria ternatea* L. blue variety in Marathi known as *gokarn* are selected for further experiment collected from Rahuri Krushi Vidyapeeth, Rahuri, Maharashtra, India.

2.2 Mutagens and Treatments
For this entire investigation *gamma rays* were used as physical mutagens, Ethyl methanesulphonate was used as chemical mutagen and the selective combination of gamma rays and EMS as combined treatment as described below.

2.2.1 Procedure for gamma rays treatment
300 healthy, uniform size and dry seeds of *Clitoria ternatea* L. blue flower variety were packed in the polythene bags and sealed for gamma radiation. Electromagnetic ionizing radiations were applied from CO^{60} source of irradiation. Gamma radiation was carried out at Nuclear Chemistry Division, Department of Chemistry, Savitribai Phule University of Pune, Ganeshkhind, Pune - 411 007. The seed samples were exposed to different doses of gamma rays like *240Gy, 300Gy, 360Gy* and *420Gy*.

2.2.2 Procedure for EMS (Ethyl methanesulphonate) treatment
Ethyl methanesulphonate ($CH_3SO_3C_2H_5$) was obtained from *Sigma Aldrich* (India) with a molecular weight 124.16 g/mol and its density 1.20g/cm^3 for the present experimental research work to determine the lethal doses of LD_{50} at suitable concentration of mutagens. Around 25±2 ℃ temperature was maintained while treating the seeds with EMS. And healthy, uniform size and dry seeds of the C*litoria ternatea* L. blue flower variety were selected for the present investigation were presoaked in distilled water for six hours to make the seed coat permeable for the chemical mutagenic treatment. And now the presoaked seeds were immersed in chosen concentrations of *0.25%, 0.50%, 0.75%* and *1%* of EMS solution for four hours under continuous stirring and washed under running tap water thoroughly for three to four times to wash out the residual EMS and soaked again in distilled water for four hours.

2.2.3 Combination treatment (γ-rays and EMS)
After gamma rays were irradiated at different doses of *240Gy, 300Gy, 360Gy* and *420Gy*, chemical mutagenic treatment of EMS was conducted on the same seed samples. The selected and combined doses and concentrations of gamma rays as EMS are *240Gy+1%, 300Gy+0.75%, 360Gy+0.50%,* and *420Gy+0.25%.*

2.3 Methodology and techniques
The seeds of *Clitoria ternatea* L. treated with γ-rays, EMS and the combination of γ and EMS used for the present investigation were sown in the experimental field by complete randomized block design (CRBD) along with control seeds with three replicates of each treatment to rise M_1 generation.

2.3.1 Seed germination percentage
From each treatment 100 seeds were used for seed germination, seedling height. Three replicates of 50 seeds per replicate kept in Petri-plate containing moist germination paper as shown in Fig. 1B. And the seed germination percentage was recorded after a week time. The total number of seeds germinated by the seventh day for each treatment along with control and the data was expressed as germination percentage as followed.

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

2.3.2 Seedling injury and seedling height
After one week of seed germination, the seedling height was recorded as shown in Fig. 1C, 1D, 1E and 1F. And then, by the method of (Mayhill and Konzak, 1967), the seedling injury was calculated from the measured values of seedling height.

2.3.3 Pollen sterility test
From each treatment and control, 10 plants were selected at random and pollen grains from freshly dehisced anthers were stained with 1% acetocarmine stain. And the partially or shriveled grains were considered as *sterile* and the stained were as *fertile* as shown in Fig. 5.

3. Experimental observations
3.1 Effect of mutagens on seed germination percentage
As shown in Table 1, seed germination percentage was decreases with increase in doses or concentrations of mutagens. Gamma rays show seed germination percentage at 240Gy with 79%, 300Gy with 75.21%, 360Gy with 73.33% and 420Gy with 65% respectively. EMS with 0.25%, 0.50%, 0.75% and 1% shows 73%, 71%, 66% and 55% seed germination respectively. Combination treatment of gamma rays and EMS, 240Gy+1% with 74.6%,

300Gy+0.75% with 72%, 360Gy+0.50% with 71%, 420Gy+0.25%with 68% seed germination respectively as shown in Figure 2. The least germination percentage of the seeds was observed in the 1% EMS treatment and the highest was observed in 240Gy treatment after control. The variation of seed germination percentage for control and treated seeds was as shown in Fig. 2.

3.2 Effect of mutagens on seedling height and seedling injury

As shown in Table 2, seedling height in gamma treatments of 240Gy was 9.83cm, 300Gy was 9.75cm, 360Gy was 8.27cm and in 420Gy it was 7.42cm. And the EMS treatments of 0.25% showed 7.65cm, 0.50% showed 6.18 cm, 0.75% showed 5.02cm and 1% with 4.47cm seedling height respectively. 1% EMS showed lowest height of seedling height. Combination treatment of 240Gy+1% showed 7.49 cm, 300Gy+0.75% with 7.31cm, 360Gy+0.50% with 6.63cm, 420Gy+0.25% with 5.74 cm highest seedling height. Among gamma treatments, 240Gy showed the less damage and highest damage was in 1% of EMS treatment. And the variation of seedling height, seedling injury and percentage of seedling injury was as illustrated in Fig. 3A and 3B.

3.3 Effect of mutagens on pollen sterility

During M_1 generation of *Clitoria ternatea* L., the pollen sterility was aggravated with the rise in the doses or concentrations of the mutagens. In Control, the pollen sterility was perceived to be 5.1%. For EMS treatments, the pollen sterility was in the range of 15.2% to 16 %. In gamma irradiated plants the pollen sterility was in the range of the 6.05% to 10.6%. In Combination treatment the pollen sterility was observed from 10.79% to 14.16% as shown in Table 3. Lowest pollen sterility observed in gamma treatment and higher pollen sterility was observed in EMS treatment. And the variation of percentage of pollen sterility was as illustrated in Fig. 4.

4. Results and Discussion

4.1 Seed germination percentage

Seed germination was impeded with the increase of the doses or concentration of the all mutagens of EMS, gamma radiation and combination treatments as shown in Fig. 2. The lowest seed germination recorded in 1% EMS treatment while the highest germination percentage observed in 240Gy treatment. (Mangaiyarkarasi *et al*., 2014) [10] published that the germination percentage was fallen off with increase in doses or concentrations of Gamma rays and EMS treatments in *Catharanthus roseus*. And seed germination percentage was also fallen off with rise in concentrations or doses of EMS, Gamma rays and combination treatments in *Lablab purpureus* L. (Jagtap and More, 2015) [6]. It was noted that mutagenic effectiveness and efficiency increased with the decreased dose or concentration. Similar finding was noted by (Ravichandran and Jaykumar, 2015) in sesame plant. They also reported that the gamma and EMS treatments produced a high frequency as well as a wide spectrum of mutations. The low germination in 1% of EMS could be as a result of physiological and severe chromosomal destruction. Delay in the onset of mitosis and chromosomal aberration induced enzymatic activity result in reduced germination (Ananthswami *et al*., 1971) [2]. The seed germination, seedling height and seedling injury, survival at maturity, plant height, and pollen fertility were reduced with increases in dose or concentration of the mutagens in *Sesanum indicum* L. was studied by (Sheeba *et al*., 2003) [18].

4.2 Seedling height and seedling injury

The inhibition of the cell division and the chromosomal anomaly are the main causes of the reduction of the seedling height and seedling injury (Reddy *et al*., 1992) [16]. In the case of *Clitoria ternatea* L., the seedling height was reduced with increase in the doses or concentrations of the chemical and physical mutagens. Seedling height initially increased for 240Gy treatment and it decreased again in the same concentration of the mutagen. And the seedling injury increased with the increase in the dose or concentration of the mutagens. The similar results were discovered by (Monica and Seetharaman, 2014) [11]. The seedling height was hindered with increase in the concentrations or doses of EMS, Gamma rays and combination treatments. The mutagenic induced reduction in seedling height in Dolichos bean was reported by (Jagtap and More, 2015) [6].

4.3 Sterility of pollens

The sterility of pollens was aggravated with the rise in the dose or concentration of mutagens. For all the mutagens of Gamma irradiation, EMS and combination, the sterility of pollens was aggravated during M_1 generation as shown in Fig. 4. The pollen sterility percentage was enhanced due to the meiotic anomaly that was induced by physical and chemical mutagens (Rana *et al*., 1964; Sinha *et al*., 1972; Mathusamy *et al*., 2002; Khan and Wani *et al*., 2005) [15, 19, 7]. The pollen sterility can be increased due to physiological and genetical changes in plants (Larik, 1975) [8]. Different researchers had identified that the pollen sterility increased with doses or concentrations of mutagens (Hakande *et al.*, 1990) [4]. Because of chromosomal anomaly, chromosomal exchange and gene mutation the percentage of pollen sterility escalated as doses or concentrations increased in both the mutagens (Gutam *et al*., 1992).

5. Tables and Figures

Table 1: Seed germination percentage in M_1 generation of *Clitoria ternatea* L.

Mutagens	Concentration/ Dose	Germination of Seeds (%)	S.E
Control	-	82	±0.40
EMS	0.25%	73	±0.32
	0.50%	71	±0.20
	0.75%	66	±0.39
	1%	55	±0.45
γ-Rays	240Gy	79	±0.50
	300Gy	75.21	±0.60
	360Gy	73.33	±0.66
	420Gy	65	±0.25
γ- Rays + EMS	240Gy+1%	74.6	±0.15
	300Gy+0.75%	72	±0.50
	360Gy+0.50%	71	±0.50
	420Gy+0.25%	68	±0.80

Table 2: Seedling height and seedling injury in M_1 generation of *Clitoria ternatea* L.

Mutagens	Concentration / Dose	Seedling Height (cm)	Seedling Injury (cm)	Seedling Injury (%)	S.E
Control		11	-	-	±0.5773
EMS	0.25%	7.65	3.35	30.45	±0.0057
	0.50%	6.18	4.82	34.70	±0.0115
	0.75%	5.02	5.98	54.36	±0.3059
	1%	4.47	6.53	59.36	±0.0173
γ-Rays	240Gy	9.83	1.17	10.64	±0.0230
	300Gy	9.75	1.25	11.36	±0.0288
	360Gy	8.27	2.73	24.89	±0.0923
	420Gy	7.42	3.58	32.54	±0.0404
γ- Rays + EMS	240Gy+1%	7.49	3.51	31.90	±0.1558
	300Gy+0.75%	7.31	3.69	33.54	±0.0635
	360Gy+0.50%	6.63	4.37	39.72	±0.1096
	420Gy+0.25%	5.74	5.26	47.81	±0.0923

Table 3: Effect of mutagens on pollen sterility in M_1 generation of *Clitoria ternatea* L.

Mutagens	Concentration/ Dose	% Pollen Sterility	S.E
Control	-	5.15	±0.5
EMS	0.25%	10	±0.01
	0.50%	15.2	±0.25
	0.75%	15.4	±0.4
	1%	16.45	±0.2
γ-Rays	240Gy	6.05	±0.2
	300Gy	9	±0.15
	360Gy	10.6	±0.1
	420Gy	10.15	±0.4
γ - Rays + EMS	240Gy+1%	10.79	±0.05
	300Gy+0.75%	11.25	±0.1
	360Gy+0.50%	13.7	±0.26
	420Gy+0.25%	14.16	±0.35

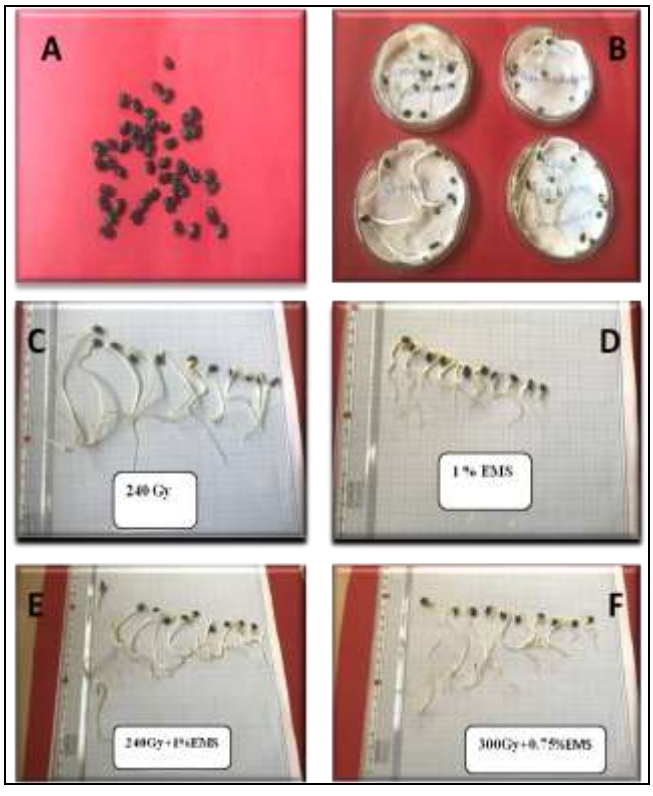

Fig 1: A) Seeds of *Clitoria ternatea* L. B) Seed germination of control and treated seeds

C) Seedling height of 240Gy treated seeds D) Seedling height of 1% EMS treated seeds
E) Seedling height of 240+1% EMS treated seeds F) Seeling height of 300Gy+0.75% treated seeds

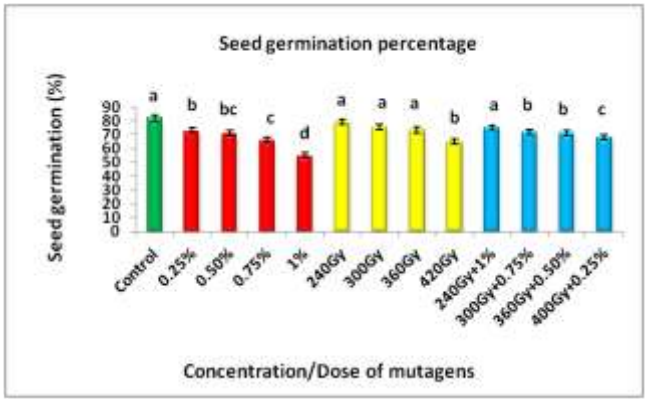

Fig 2: Effect of mutagens on seed germination in M_1 generation of *Clitoria ternatea* L.

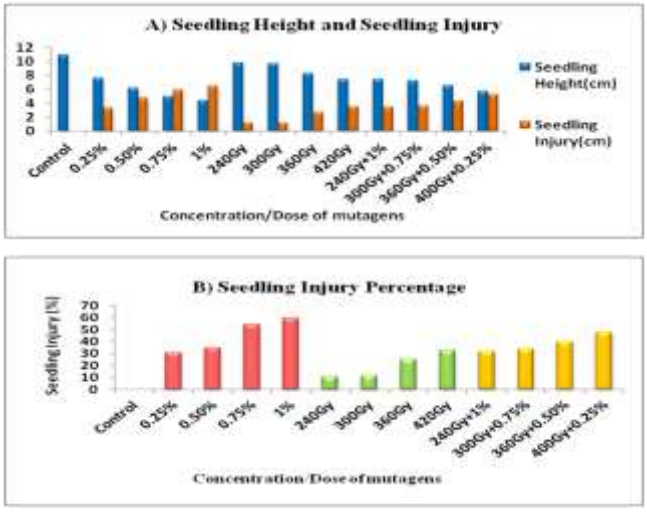

Fig 3: A) Effect of mutagens on seedling height and seedling injury in M_1 generation of *Clitoria ternatea* L. B) Effect of mutagens on seedling injury percentage in M_1 generation of *Clitoria ternatea* L.

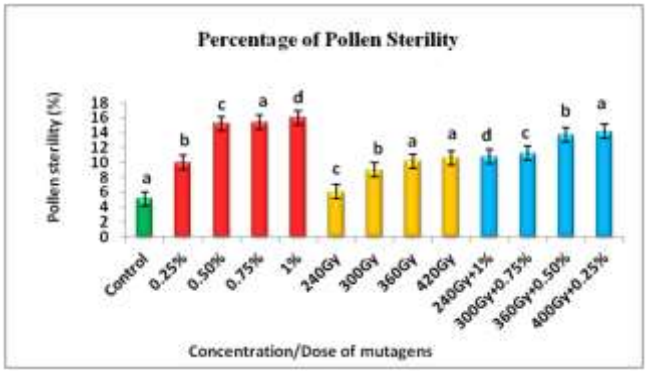

Fig 4: Effect of mutagens on pollen sterility in M_1 generation of *Clitoria ternatea* L.

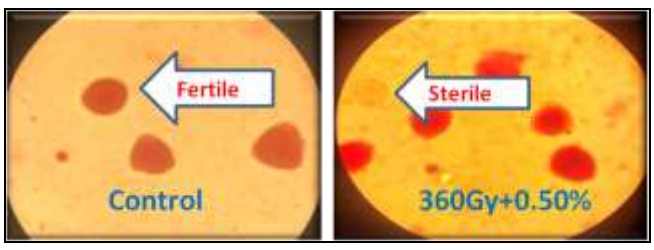

Fig 5: Fertile and Sterile pollens of *Clitoria ternatea* L.

6. Conclusions

The raise in the concentrations of Ethyl methanesulphonate and dose of γ irradiation empirically perceived the decrease in the percentages of seed germination, and seedling height. And the pollen sterility was enhanced in 1% of EMS treatment and least in 240Gy among the treated plants. Therefore it can be very well concluded that both the mutagens indicated a hindered effect on seed germination, seedling injury and pollen sterility. Hence, this study reveals the optimal mutagenic doses needed for the generation of *Clitoria ternatea* L. with qualitative and quantitative genetically varied traits.

7. Acknowledgement

I'm grateful to Dr.R.G.Pardeshi, Principal, Fergusson College, Dr.A.D. More, Associate Professor, Dept. of Botany, Fergusson College, Pune, India, Mrs. S.S Kate, Head, Department of Botany, Fergusson College and Prof. A.B. Ade, Head, Department of Botany, Savitribai Phule Pune University, Pune, for providing required facilities and encouragement for my research work.

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