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**SR Merrin Rabinya**
Department of Genetics and Plant Breeding, TNAU, Coimbatore, Tamil Nadu, India

**C Babu**
Directorate of Research, TNAU, Coimbatore, Tamil Nadu, India

**T Ezhilarasi**
Department of Forage Crops, CPBG, TNAU, Coimbatore, Tamil Nadu, India

**V Manivannan**
Department of Agronomy, DCM, TNAU, Coimbatore, Tamil Nadu, India

**V Ravichandran**
Department of Crop Physiology, DCM, TNAU, Coimbatore, Tamil Nadu, India

**Corresponding Author:**
**C Babu**
Directorate of Research, TNAU, Coimbatore, Tamil Nadu, India

# Induction of gamma ray mutants for assessing the lethality and spectrum of chlorophyll mutations in $M_1$ and $M_2$ progenies of fodder cowpea [*Vigna unguiculata* L (Walp)]

**SR Merrin Rabinya, C Babu, T Ezhilarasi, V Manivannan and V Ravichandran**

**Abstract**
Seeds of fodder cowpea varieties CO 8 and CO 9 were irradiated with different doses of Gamma rays from 100 Gy to 2000 Gy for the purpose of creating variability and broaden the genetic basis. The treated seeds were raised in seedling trays where the survival rate was nearly zero at the doses from 1600 - 2000 Gy. Lethal Dose 50 ($LD_{50}$) values were determined using probit analysis based on the germination (or) mortality percent as 658.52 Gy for CO 8 and 626.82 Gy for CO 9 and effective doses were fixed *viz.*, 559 Gy, 659 Gy and 759 Gy for CO 8 and 527 Gy, 627 Gy and 727 Gy for CO 9. Study on $M_1$ generation was carried out and parameters like germination percentage at seventh day after sowing, seedling shoot length, seedling root length, seedling vigor index, survival rate on 30$^{th}$ day after sowing, pollen fertility and quantitative traits like plant height at 30$^{th}$ day after sowing, leaf length, leaf breadth and number of primary branches at fodder harvesting stage were recorded. These seedling parameters and quantitative traits exhibited a decreasing trend along with the increasing doses of gamma rays which resulted in considerable variability. In $M_2$ generation, the presence of chlorophyll mutants was observed and these mutants include *Albina*, *Chlorina*, *Xantha*, *Viridis*, *Xantha viridis* and *Aurea* which occurred in different relative frequencies in the corresponding two fodder cowpea varieties. The chlorophyll mutation *Aurea* occurred at a very low frequency in both the varieties CO 8 and CO 9. These decreasing trends in seedling parameters and quantitative traits and the occurrence of chlorophyll mutants represent the potency and efficiency of the mutagen. It was observed that the seeds of variety CO 9 were more sensitive to gamma rays than CO 8.

**Keywords:** $LD_{50}$, fodder cowpea, gamma rays, $M_1$ & $M_2$ generation, chlorophyll mutants

## Introduction
India possesses the world's largest livestock sector, housing 20% of the global livestock population and standing as the leading milk producer. The livestock population in the country has reached 536.76 million, marking a growth of 4.8% compared to the 19$^{th}$ Livestock Census (2012). According to the 20$^{th}$ Livestock Census 2019 [1], the total bovine population (cattle, buffalo, mithun and yak) accounted to 303.76 million, demonstrating 1.3% increase over the previous census and this increase in livestock population faced a deficit of 11.24% for green fodder where the total green fodder availability is 734.19 mt against the requirement of 827.19 mt (Roy *et al*., 2019) [19]. The primary solution for closing the significant disparity between fodder supply and demand is to optimize the production of fodder per given unit of land and time within the current agricultural farming systems.

Cowpea [*Vigna unguiculata*. L (Walp)], 2n=2x=22 is predominantly cultivated in semi-arid regions of Latin America, Africa and South Asia serving a dual purpose as a vital source of food for both humans and livestock. The term "hungry-season crop" is attributed to cowpea due to its status as the initial crop to be harvested, preceding cereal crops (Gomez, 2004) [8]. The drought tolerance nature of the crop allows successful growth in challenging conditions. The crop thrives even in poor sandy soils with limited organic and phosphorus content. This legume holds greater importance in tropical and subtropical countries, providing essential grains, vegetables and animal fodder (Kumar *et al.*, 2022) [12]. Fodder cowpea is well-suited for integration into a Food-Fodder cropping system due to its short crop duration. Additionally, it could be recommended even in rainfed conditions. Sine cowpea is a self-pollinating crop, its genetic variability and diversity are limited (Boukar *et al.*, 2019) [3] in the

development of high biomass cowpea. Hence mutation breeding can be deployed to improve green fodder yield and fodder quality traits thus ensuring the supply of protein-rich supplement to livestock. Additionally, mutagenesis increases variability, which may allow for the reemergence of long-lost traits (Khursheed *et al.,* 2019) [10].

Mutation breeding, a cutting-edge approach to plant breeding, is a quick, affordable, and cogent way to speed up the process of creating and screening crop genotypes with unique and improved agronomic traits. Fixing the optimum mutagen dose is a key factor in the success of mutation breeding. Maximum mutation and minimal lethality are produced by a mutagen at its optimal dose. Accordingly, $LD_{50}$ is a significant factor that helped plant breeders to discover the most effective mutagen doses for inducing mutations (Alvarez-Holguin *et al.,* 2019) [2]. Therefore, the main objective of this study is to estimate the lethal dose ($LD_{50}$) and further analysis of seedling parameters and leaf component traits $M_1$ generation and the spectrum of chlorophyll in $M_2$ generation.

## Materials and Methods

Two fodder cowpea varieties CO 8 and CO 9 were chosen to induce mutation by the physical mutagen, Gamma rays. Seeds of those two varieties were collected from the Department of Forage Crops, Center for Plant Breeding and Genetics, TNAU, Coimbatore.

For fixing $LD_{50}$ value, gamma irradiation was carried out in the "Co60 Gamma Chamber 5000" at the Indira Gandhi Center for Atomic Research (IGCAR), Kalpakkam, Chennai, where the source of gamma irradiation $^{60}Co$ (Cobalt) was emitted at the rate of 28 Gy/min dose in every seed and the facility was used in Radiological Safety Division. The seeds of two fodder cowpea varieties were exposed to 20 doses from 100 to 2000 Gy with 100 Gy interval in which each treatment consisted of 50 well filled seeds which were raised in 50 cell type seedling trays with two replications of 25 seeds each along with control. Germination percent (%) was calculated on 7$^{th}$ days after sowing. Then, survival rate (%), seedling vigor index, root length (cm) and shoot length (cm) were measured on 21$^{st}$ days after sowing.

The $LD_{50}$ values of gamma radiation for both the varieties were determined based on the probit analysis. The probit function is the inverse Cumulative Distribution Function (CDF) or quantile function associated with the standard normal distribution. The procedure for determination of $LD_{50}$ using probit analysis is that the dosage values for mutagens were converted into their $\log_{10}$ equivalents. The mortality rate of seeds resulting from the treatment doses were calculated and rounded to the nearest whole number. The adjusted mortality percentage was computed using the below mentioned Abbott's formula and rounded to the nearest whole number.

$$\text{Corrected mortality (\%)} = \frac{M_{observed} - M_{control}}{100 - M_{control}} \times 100$$

The adjusted figures were transformed into the probit scale. A graph was created with probit values on the Y-axis and $\log_{10}$ concentration on the X-axis. A straight line was drawn through the majority of the plotted points to estimate the $\log_{10}$ concentration, which corresponds to a probit value of 5. The antilogarithm of the $\log_{10}$ value corresponding to probit 5 was computed to determine the $LD_{50}$ for Gamma rays.

The experiment was set up in randomized block design (RBD) for the $M_1$ generation consisting of the effective doses *viz.,* 559 Gy, 659 Gy and 759 Gy for CO 8 and 527 Gy, 627 Gy and 727 Gy for CO 9, where 300 seeds were sown in three replications (100 seeds each) along with control. The spacing adopted was 45 x 15 cm. Germination percent (%), root length (cm) and shoot length (cm) were the observations measured on 7$^{th}$ day. Survival rate (%) and plant height (cm) were measured on 30$^{th}$ day. Pollen fertility (%) was examined during flowering stage and biometrical traits like leaf length (cm), leaf breadth (cm) and number of primary branches were taken during fodder harvesting stage in 10 randomly selected plants for each replicate of the treatment.

Ten flowers selected at random for each replicate of the treatment were utilized for studying pollen fertility (%). It was observed by staining the newly dehisced anthers with 1% solution of potassium iodide. Pollen grains that exhibited staining were classified as fertile, while those that appeared shrunken and unstained were designated as sterile. Then the number of stained and unstained pollen grains were counted, thus evaluating pollen fertility by using the formula,

$$\text{Pollen fertility (\%)} = \frac{\text{Number of stained pollens}}{\text{Total number of pollens observed}} \times 100$$

Seedling Vigor Index (SVI) can be calculated by using the formula,

SVI = Germination% x [Mean root length+ Mean shoot length]

Thus, all the seedling parameters and some quantitative characters were observed and analyzed and $M_2$ generation was raised for studying the spectrum of chlorophyll mutations.

## Results and Discussion

$LD_{50}$ values for Gamma rays were calculated with the aid of probit analysis based on the germination raised in seedling trays. The optimum dose is the dose that causes a maximum of mutation with a minimum of damage to the plant (Ramchander *et al*., 2015[16]; Veni *et al*., 2017 [24]). The nature of mutation is decided by determining the correct doses and concentration of mutagens which can be determined by establishing the $LD_{50}$ value for the mutagen to be used. Since the $LD_{50}$ value is genotype dependent, the value has to be decided for each of the genotypes to be mutagenized. In the present study, the $LD_{50}$ value for CO 8 was 658.52 Gy (Table 1 and Fig. 1) and for CO 9 it was 626.82 Gy was registered (Table 1 and Fig. 2). Similarly, Olasupo, *et al*., (2016) [14] has reported wide range of $LD_{50}$ value of 329 to 1054 Gy in cowpea based on seed germination percentage.

**Table 1:** Determination of lethal doses of Gamma rays in CO 8 and CO 9 by Probit analysis

| Dose (Gy) | % Mortality | | Corrected mortality | | Log dose (x) | | Empirical Probit | | $LD_{50}$ | |
|---|---|---|---|---|---|---|---|---|---|---|
| | CO 8 | CO 9 | CO 8 | CO 9 | CO 8 | CO 9 | CO 8 | CO 9 | CO 8 | CO 9 |
| 0.00 | 0 | 0 | 0.0 | 0.0 | | | | | 658.52 (Gy) | 626.82 (Gy) |
| 100.00 | 14.00 | 20.00 | 14.0 | 20.0 | 2.00 | 2.00 | 3.92 | 4.16 | | |
| 200.00 | 12.00 | 28.00 | 12.0 | 28.0 | 2.30 | 2.30 | 3.83 | 4.42 | | |
| 300.00 | 24.00 | 16.00 | 24.0 | 16.0 | 2.48 | 2.48 | 4.29 | 4.01 | | |
| 400.00 | 28.00 | 30.00 | 28.0 | 30.0 | 2.60 | 2.60 | 4.42 | 4.48 | | |
| 500.00 | 36.00 | 36.00 | 36.0 | 36.0 | 2.70 | 2.70 | 4.64 | 4.64 | | |
| 600.00 | 46.00 | 52.00 | 46.0 | 52.0 | 2.78 | 2.78 | 4.90 | 5.05 | | |
| 700.00 | 48.00 | 44.00 | 48.0 | 44.0 | 2.85 | 2.85 | 4.95 | 4.85 | | |
| 800.00 | 58.00 | 52.00 | 58.0 | 52.0 | 2.90 | 2.90 | 5.20 | 5.05 | | |
| 900.00 | 60.00 | 76.00 | 60.0 | 76.0 | 2.95 | 2.95 | 5.25 | 5.71 | | |
| 1000.00 | 64.00 | 78.00 | 64.0 | 78.0 | 3.00 | 3.00 | 5.36 | 5.77 | | |
| 1100.00 | 66.00 | 66.00 | 66.0 | 66.0 | 3.04 | 3.04 | 5.41 | 5.41 | | |
| 1200.00 | 72.00 | 64.00 | 72.0 | 64.0 | 3.08 | 3.08 | 5.58 | 5.36 | | |
| 1300.00 | 70.00 | 62.00 | 70.0 | 62.0 | 3.11 | 3.11 | 5.52 | 5.31 | | |
| 1400.00 | 72.00 | 60.00 | 72.0 | 60.0 | 3.15 | 3.15 | 5.58 | 5.25 | | |
| 1500.00 | 76.00 | 64.00 | 76.0 | 64.0 | 3.18 | 3.18 | 5.71 | 5.36 | | |
| 1600.00 | 76.00 | 74.00 | 76.0 | 74.0 | 3.20 | 3.20 | 5.71 | 5.64 | | |
| 1700.00 | 80.00 | 72.00 | 80.0 | 72.0 | 3.23 | 3.23 | 5.84 | 5.58 | | |
| 1800.00 | 80.00 | 82.00 | 80.0 | 82.0 | 3.26 | 3.26 | 5.84 | 5.92 | | |
| 1900.00 | 86.00 | 82.00 | 86.0 | 82.0 | 3.28 | 3.28 | 6.08 | 5.92 | | |
| 2000.00 | 90.00 | 92.00 | 90.0 | 92.0 | 3.30 | 3.30 | 6.28 | 6.41 | | |

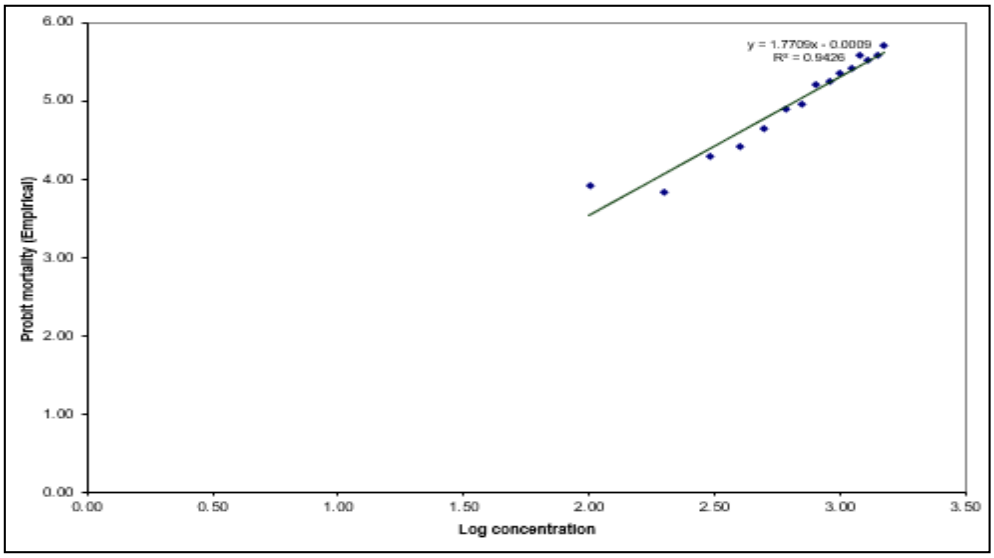


**Fig 1:** Plots of Log doses versus probits from Table 1 for CO 8

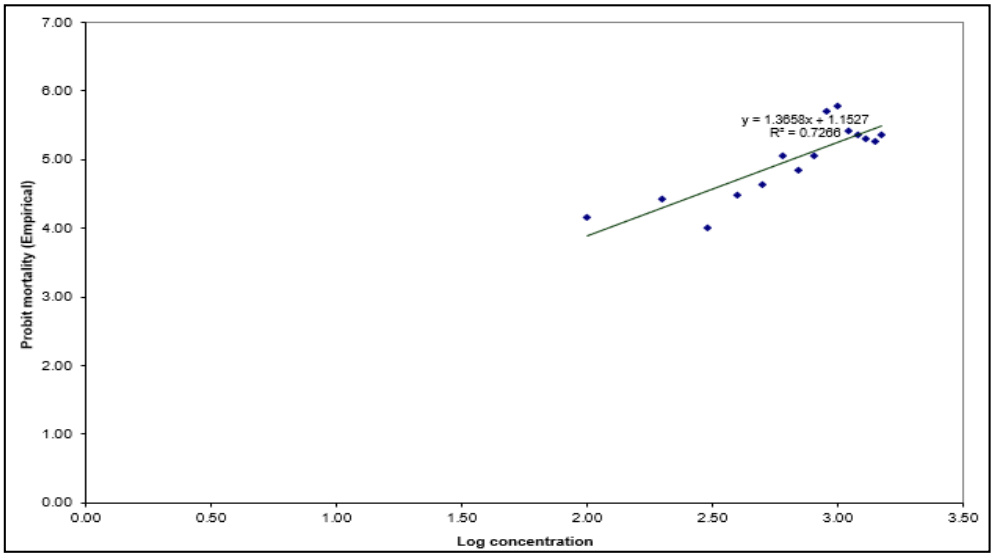


**Fig 2:** Plots of Log doses versus probits from Table 1 for CO 9

**$M_1$ generation**

The potency of the mutagen can be evaluated by studying the biological damages and chlorophyll mutations produced in $M_1$ and $M_2$ generation respectively (Eswaramoorthy *et al*., 2021) [5]. In the present study, the percentage of seed germination, decreased progressively with increasing dose of physical mutagen in both the varieties under field conditions (Table 2).

**Table 2:** Effect of gamma rays in $M_1$ generation of fodder cowpea CO 8 and CO 9

| Variety | Trt (Gy) | Total no. of seeds | Mean germination (%) | Mean shoot length (cm) | Mean root length (cm) | Seed vigor index | Mean survival on 30th day (%) | Plant height on 30th day (cm) | Pollen fertility (%) | Leaf length (cm) | Leaf breadth (cm) | Number of primary branches |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| CO 8 | Ctrl | 300 | 96.67 | 10.93 | 13.45 | 2356.8 | 91.67 | 31.49 | 93.6 | 10.78 | 7.22 | 9.33 |
| | 559 | 300 | 74.6 | 9.81 | 12.71 | 1679.9 | 68 | 30.36 | 54.3 | 8.28 | 6.1 | 7.47 |
| | 659 | 300 | 53.3 | 7.98 | 10.37 | 978.05 | 49.3 | 28.02 | 37.9 | 7.54 | 5.98 | 6.93 |
| | 759 | 300 | 29.3 | 6.67 | 9.11 | 462.3 | 23.3 | 26.18 | 20.3 | 6.7 | 5.74 | 6.46 |
| CO 9 | Ctrl | 300 | 97.33 | 9.49 | 12.87 | 2176.3 | 93.33 | 35.67 | 94.3 | 10.99 | 8.32 | 9.8 |
| | 527 | 300 | 74.67 | 9.18 | 11.32 | 1530.73 | 69.6 | 33.49 | 51.3 | 8.59 | 6.31 | 8.33 |
| | 627 | 300 | 54 | 7.61 | 9.04 | 899.1 | 48.3 | 32.65 | 31.8 | 8.41 | 6.28 | 5.67 |
| | 727 | 300 | 24.67 | 7.27 | 8.58 | 391.01 | 20.6 | 30.07 | 21.7 | 7.48 | 5.64 | 4.67 |

Biological damages resulting from mutations in germination (%), survival rates (%), plant height (cm) at 30 days after sowing, pollen sterility (%) and plant height (cm) at maturity can serve as indicators of mutagenic influence, as suggested by Gaul (1964) [6]. The reduction in seed germination at elevated mutagen doses might be due to the disturbance of seed layers or outer covering, cytological harm and modifications in metabolic processes involving both breakdown and synthesis. The α and β amylase enzymes play a role in breaking down starch into sugars, which fuel the growth of roots and shoots as seeds sprout. Alterations in the activity of these enzymes due to mutagenic effects could lead to a decrease in germination percentage (Kulkarni, 2011) [11]. The maximum reduction in seed germination percent was 29.3% in CO 8 at 759 Gy and 24.67% in CO 9 at 727 Gy. The effect of mutagen on root and shoot development was studied on seventh day after sowing. The reduction in root and shoot length may be due to hampered protein synthesis in embryonic cells which resulted in prevention of cell passage from the G1 phase leading to retardation in root and shoot development (Casarett, 1968) [4].

The seedling vigor index (SVI) is another important seedling parameter which determines the overall growth activity of epicotyl and hypocotyl within the embryo inside the seed. Seedling vigor index exhibited maximum inhibition in CO 8 at 759 Gy when compared with CO 9 at 727 Gy. Cytogenetic harm and physiological abnormalities are thought to be the causes of decreased plant survival (Sato and Gaul, 1967) [21]. The maximum reduction in survival was recorded in higher doses of gamma rays in both varieties. The vigor index exhibited a gradual decrease as the mutagenic doses increased. The results revealed that, a decreasing tendency for plant height as mutagen doses increased. According to Swaminathan *et al.* (1962) [23], the biological effects of a mutagen on the $M_1$ generation are frequently assessed using an indicator of plant damage. The height of the seedlings was observed to be retarded by all treatments. The percentage of pollen fertility showed an inverse relationship with the increasing levels of gamma irradiation (Fig.3 and Fig.4). The decrease in pollen fertility might be due to the cumulative impact of various abnormal stages of meiosis, as well as physiological and genetic damage possibly caused by chromosome breakage, which leads to the production of anti-metabolic substances within cells. Alternatively, it could result from the improper separation of chromosomes during the anaphase stage (Larik, 1975) [13].

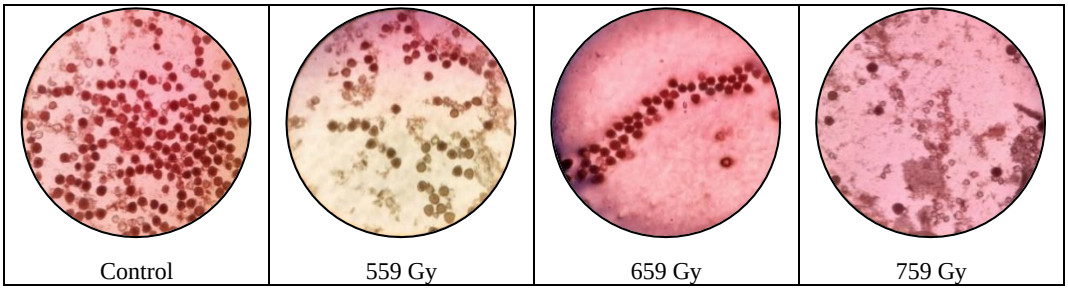


**Fig 3:** Effect of gamma rays on pollen fertility in CO 8

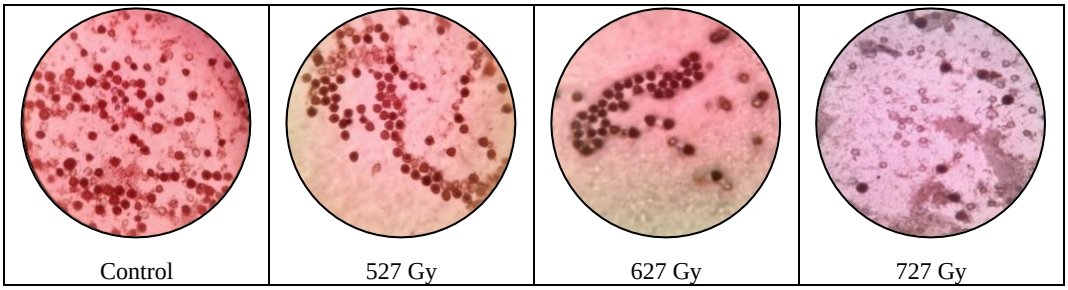


**Fig 4:** Effect of gamma rays on pollen fertility in CO 9

Quantitative traits like leaf length (cm), leaf breadth (cm) and number of primary branches exhibited gradual biological damage representing the potency of gamma rays in inducing variability.

**$M_2$ generation**

In $M_1$ generation, all the survived plants were harvested on a single plant basis and forwarded to $M_2$ generation in progeny rows and the presence of chlorophyll mutants were observed.

**Chlorophyll mutations**

Chlorophyll mutations provide one of the most dependable indices for evaluations of genetic effects of mutagenic treatments and have been reported in various pulse crops by several workers (Pandey and Dhanasekar, 2004)[15].These chlorophyll mutations are used as genetic markers in basic and applied research (Reddy and Gupta,1989)[18]. The seedling's chlorophyll mutation was assessed in the field from the day of emergence till fourth week of the $M_2$ generation. In the present study, *chlorina* type chlorophyll mutant occurred most frequently followed by other mutations like *xantha*, *viridis*, *albina*, *aurea* and *xantha viridis* (Table 3 and Fig.5 and 6).

**Table 3:** Spectrum and frequency of different chlorophyll mutations in $M_2$ generation of *Vigna unguiculata* var. CO 8 & CO 9

| Variety | Dose (Gy) | Spectrum of chlorophyll mutations | | | | | | Frequency of chlorophyll mutations | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *Albina* | *Chlorina* | *Xantha* | *Xantha viridis* | *Viridis* | *Aurea* | *Albina* | *Chlorina* | *Xantha* | *Xantha viridis* | *Viridis* | *Aurea* |
| CO 8 | 559 | 7 | - | - | 8 | - | 9 | 0.34 | - | - | 0.39 | - | 0.44 |
| | 659 | 3 | - | - | 5 | - | 4 | 0.20 | - | - | 0.33 | - | 0.27 |
| | 759 | 1 | - | - | 2 | - | 2 | 0.14 | - | - | 0.29 | - | 0.29 |
| CO 9 | 527 | - | 12 | 8 | - | 7 | 5 | - | 0.57 | 0.38 | - | 0.33 | 0.23 |
| | 627 | - | 7 | 5 | - | 4 | 3 | - | 0.48 | 0.23 | - | 0.27 | 0.20 |
| | 727 | - | 2 | 2 | - | 1 | 1 | - | 0.32 | 0.32 | - | 0.16 | 0.16 |

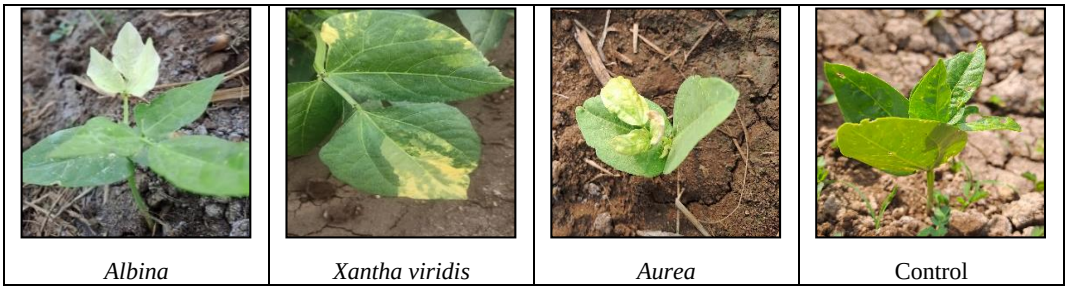


**Fig 5:** Chlorophyll mutations in CO 8

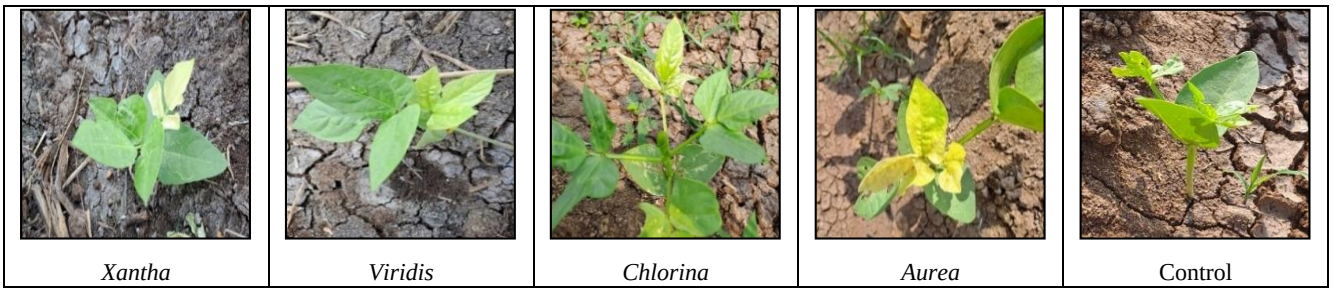


**Fig 6:** Chlorophyll mutations in CO 9

The development of chlorophyll seems to be under the control of numerous genes located on various chromosomes (Goud, 1967) [9], potentially situated adjacent to centromeres and proximal segments of the chromosome (Swaminathan *et al.*, 1964) [22]. Ramulu *et al.*, (1970) [17] suggested that differences in the mutation spectrum and rate among different genotypes may arise due to variations in the location of chlorophyll genes in relation to the centromere. The occurrence of *chlorina* mutants has been attributed to different causes, such as impaired chlorophyll biosynthesis, further degradation of chlorophyll and bleaching due to a deficiency of carotenoids. This implies that genetic differences in the genotypes studied for inducing chlorophyll mutation types have been observed, as identified by several researchers (Giri and Apparao, 2011 [7] and Sangle and Kothekar *et al.*, 2013 [20]) in pigeon pea.

**Conclusion**

Estimating induced biological damage aids in assessing the sensitivity of a biological material as well as the potency of a mutagen. The increasing concentrations of mutagens hindered the percentage of seed germination and the growth of seedlings. Germination parameters such as germination percent, root length, shoot length and vigor index exhibited a decreasing trend with the elevated mutagen dose under seedling tray conditions. A similar result was also reported under field conditions for both germination percentage and survival rate. The spectrum of chlorophyll mutations was *xantha viridis*>*albina*>*aurea* in fodder cowpea CO 8 and in case of CO 9 it was *chlorina*>*viridis*>*xantha*>*aurea.* It was observed that the seeds of variety CO 9 were more sensitive to gamma rays than CO 8.

**Conflict of interest:** none

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