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Analyzing frequency and spectrum of chlorophyll mutation induced through Gamma ray and Combination treatment (Gamma + EMS) on genetic paradigm of *Artemisia annua* L.

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Abstract. For the development of genetic programs with novel characteristics induced mutagenesis has been used extensively. Chlorophyll (chl) mutations are considered as the most dependable indices for assessing the efficiency of different mutagens in inducing the genetic variability in crop plants and are also used as genetic markers in basic and applied research. In the present scenario of high health susceptibility, the global demand for natural medicine derived from plant species has increased enormously. Sweet wormwood (*Artemisia annua* Linnaeus) – an important medicinal plant species with immense remedial values, was selected for the present study and exposed to gamma rays at 100 Gy, 200 Gy, 300 Gy and combination treatments with 100 Gy + 0.1%EMS, 200 Gy + 0.1% EMS, 300 Gy + 0.1%EMS. Meiotic study was also done and various cytological aberrations were observed in M_2 generation like stickiness, precocious, scattering, laggard and bridge etc. The frequency of induced chl mutation varied in different mutagen treatments. Eight different types of chl mutants namely albina, chlorina, xantha, aurea, viridis, yellow viridis and tigrina etc. were recorded in M_2 generation on plant population basis. The frequency of xantha mutants was quite high in both the treatments but in gamma exposed set it was followed by albina whereas in combination treatments viridis was second highest mutant. In different mutants quantitative analysis of chl pigments was also done and content was highest in viridis i.e. 3.86µg/ml FW and lowest in albina i.e. 0 µg/ml FW . Although chlorophyll mutations thought to be lethal in nature, but present study has proven to be a milestone in identifying the threshold dose of a mutagen that would increase the genetic variability and induces new trait in *Artemisia annua* Linnaeus.

Keywords: *Artemisia annua* L. (Linnaeus), chlorophyll mutants, cytological anomalies, gamma rays, EMS.

INTRODUCTION

The medicinal plant *Artemisia annua*, also known as Sweet Wormwood or Sweet Annie, is one of the top 10 pharmaceutical crops which are getting intensive worldwide scientific consideration as this valuable treasure is the only source for the commercial pharmaceutical production of the ses-

quiterpene lactone artemisinin (Prasad and Das 1980). Artemisia has been applied in the traditional medicine, for the treatment of diabetes, depression, insomnia and stress, to clear the lymphatic system and in the oncotherapy. The whole plant of *A. annua* L.is still the most economic source of artemisinin, and the developments of high-producing plants of *A. annua* L. appear to be the main direction to obtain large quantities of relatively inexpensive artemisinin. For any successful crop improvement programme, genetic variability plays an important role because it provides a spectrum of variants for effective and better selection which can be obtained using mutation, hybridization, recombination and selection processes (Dhumal and Bolbhat 2012). Mutational breeding involves high energy radiation such as X, β and γ-rays, which are electromagnetic radiations that initiate or inhibit the growth and differentiation of plant cells and organs (Hasbullah et al. 2012) they could also modify physiological characteristics of plant to create new mutants for production of high amounts of commercially important metabolites. Ionizing radiation has been recognized as a powerful technique for plant improvement of medicinal plants (Vardhan and Shukla 2017). This technique creates genetic variability in plants, which can be screened for desirable characteristics. Previously, Koobokurd et al. (2008) reported a method for establishing in vitro plantlet variants of *A. annua* using low-dose gamma irradiation. By using gamma rays many high yielding mutant varieties have been developed world wide, which are resistant to biotic and abiotic stresses with improved quality (IAEA 2017). The success of mutation breeding programme largely depends on selection of promising mutants based on phenotypic characters (Arisha et al. 2015). EMS, as a chemical mutagen, can be used as a supplementary approach to improve desired identifiable characters such as yield related characters (Botticella et al. 2011). Chemical mutagens are not only mutagenic themselves but also affect mutation in specific ways when combined with radiation (Reddy and Smith 1981). It produces random point mutations in genetic material. So, mutation frequency, detected using various techniques, displays a wide range of variation in combination treatment where plants seeds exposed to physical mutagen followed by chemical mutagen. During M1 generation, probably identification of recessive character is difficult only mutations of dominant characters can be identified. In the M2 generation, the mutation will segregate to create homozygotes for recessive or dominant alleles (Page and Grossniklaus 2002). The most effective way to identify the phenotypic mutation is Visual screening which can be used as a primary indicator to select plants that have desired characters, for example: disease resistance, flowering earliness, plant height or growth period (Østergaard and Yanofsky 2004). Gene mutations influencing the green coloration of photosynthetically active parts are among the most common spontaneous or induced alterations arising in higher plants (Kolar et al. 2011). Although chlorophyll mutations are generally not useful for plant breeding purpose because of not having any economic value due to their lethal nature, their study could be useful in identifying the suitable mutagen and threshold dose of mutagen that would increase the genetic variability and number of economically useful mutations in the segregating generations (Wani and Anis 2004). The chlorophyll mutation frequency is an indicator to predict the frequency of factor mutations and thus an index for evaluation of genetic effects of mutagens (Walles 1973). In addition, chl mutations are important for identifying gene function and elucidation of chl metabolism and its regulation[15]. The occurrence of chl mutations after treatments with physical and chemical mutagens have been reported in several crops (Swaminathan et al. 1962; Sharma and Sharma 1981; Reddy and Gupta 1989; Mitra 1996; Kharkwal 1998; Solanki 2005; Wu et al. 2007). Induced mutations can rapidly create variability in quantitatively and qualitatively inherited traits in crops. Genetic variability has been induced through mutagenesis in several plants, but the information available in *A. annua* L. is meager. In the present study attempt has been made to understand the comparative response of physical and chemical mutagens on *A. annua*, with a view to determine the mutagen and treatment causing maximum chl mutations in M2 generation and also on cytological parameter.

MATERIALS AND METHODS

Plant materials

M_2 seeds generated from the M_1 generation of variety EC-415012, were used in this study. The M_1 seeds were produced by exposing separate 1000 dry seed samples (for each dose) to 100Gy, 200Gy and 300Gy at a dose rate of 15.48 Gy/min of gamma radiation using a ^{60}Co (Cobalt 60) gamma source under ambient conditions at the National Botanical Research Institute (NBRI), Lucknow and for combination treatment concentration of Ethyl Methyl Sulphonate (EMS) solution of 0.1% was prepared. EMS solution was settled in a 0.1M phosphate buffer at pH 7.0 to avoid rapid hydrolysis (Bosland 2002). Gamma ray treated (100Gy, 200Gy, 300Gy) seeds were presoaked in water for 6h

then treated with the above-mentioned concentration of EMS at 20°C with orbital shaking(110rpm) along with control (untreated) seeds. Seeds were then thoroughly washed under running water then transferred to Petridishes containing wet filter paper and kept in a growth chamber at 25°C in the seed germinator for germination (at 2 days after the treatment).Control seeds were exposed to the same conditions except for the EMS treatment.

Experimental plan and procedure

The experiments were carried out in the first week of the month january at Roxburg Botanical Garden, Department of Botany. The M_1 plants are individually harvested and sown as M_2 families. according to the Pedigree Method; the M_1 plants are individually harvested and plants with probable mutants following phenotypic observations as plant habit variation in leaves (chl mutants), early plant vigour (poor, good and very good), plant height (short stature, up to top of the plant), sown as M_2 families .Sweet wormwood M_2 lines were grown in the field (geographical location is 25°27'43.01"N, 81°51'10.42"E) in randomized complete block design (RCBD) and allowed to produce the M_2 seeds.. The net plot size was 4 m _ 4 m, with nine rows (each 4 m long) with a 45 cm distance between two rows and approximately 20 cm distance between two plants. The untreated seeds (control) were planted in the first row of each plot. For weed control plots were irrigated during vegetative growth and the plants were harvested individually at full maturity. Germination (%) taken after 7 days and plant survival (%) was recorded after 14 days for each mutagenic treatment as well as control in M_2 generation. After a month Six phenotypic traits were analysed and recorded as plant height (cm), internodal length (cm), leaf area(per m^2), No. of primary branches, days to 50% flowering and days to maturity etc.

Cytological investigations

For the cytological analysis young floral capitula of control and variant plant of *Artemisia annua* L. with appropriate size were fixed in Carnoy's fixative (Alcohol 3: Glacial Acetic Acid 1) for 24 hrs and then transferred in 90% alcohol to preserve the capitula for meiotic study. Anthers were teased and stained in 2% acetocarmine, followed by squash preparation. Slides were observed under the microscope and pollen fertility was evaluated by acetocarmine stainability test. The snapshots of chromosomes were captured by the help Pinnacle PCTV software. For pollen fertility, mature capitula having pollen grains were dusted over glass slide and stained with acetocarmine and mounted with glycerine. Then observed under optical microscope to count the frequency of fertile and sterile pollen grains.

$$\text{Pollen fertility (\%)} = \frac{\text{No. of fertile pollen}}{\text{Total no. of pollen}} \times 100$$

Quantification of Photosynthetic pigments

Photosynthetic pigment was quantified according to Lichtanthelar and Welburn (1983)method. 20mg of leaves were taken and dissolved in 5ml of 80%acetone. Solution was extracted and were centrifuged at 15000 rpm for 10min at 10°C. The supernatant volume was diluted with 80% acetone. O.D. was taken at three different wavelength i.e. 470nm, 663nm and 646nm in the spectrophotometer and finally chl *a,* chl *b* calculated.

Observations recorded and statistical procedure

The M_2 generation was screened for phenotypic variations from germination to harvesting. The frequency of the mutant plants out of the total number of individuals in M_2 generation was calculated. The mutagenic frequency was estimated as the percentage of segregating M1 plant progenies. Chl mutations were classified into various types based on the method followed by Gustafsson(1940). For statistical analysis in the table, three replicates for each treatment were used. Statistical analysis was performed using the SPSS 16.0 software. A oneway analysis of variance (ANOVA) and Duncan's Multiple Range Test (DMRT, $P < 0.05$) was conducted for mean separation and the graph was plotted by using sigma plot 10.0 software. Actual mean and standard error were calculated and the data was subjected to analysis of variance.

RESULTS

Germination and Survival percentage

Fig. 1 shows that germination percentage and plant survival were significantly declined as the mutagen-concentration increased. Conspicuous variations were recorded after both the treatment in sweetworm wood. Athigher doses germination and survival percentage was found to be 76.60%, 61.76% (in Gamma ray) and 33.83%,24.66% (in Gamma+ EMS) respectively.

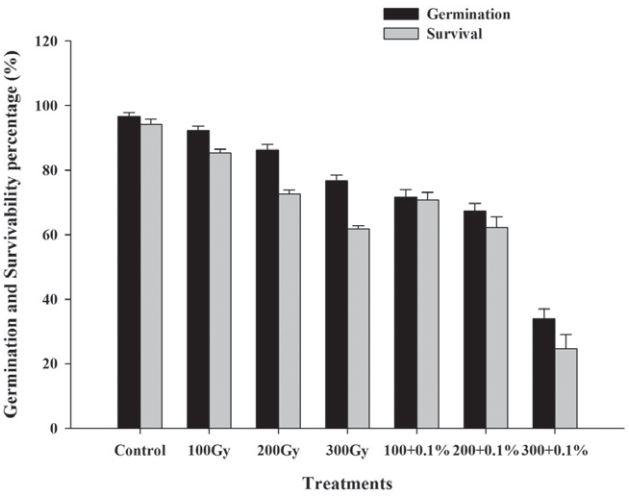

Figure 1. Effect of Gamma ray and Combination treatment on germination and survival in *Artemisia annua* L. (M1 generation).

Chl leaf variations

In the present appraisal, the frequency of chl mutants calculated as percent of M1 plant progenies and M2 plants basis were presented in Table 1. It was observed that the frequency of induced chl mutant was increased with an increase in the dose of gamma ray and combination treatment but at the maximum doses mutation frequency was decreased. At the 300Gy mutation frequency was recorded as 3.60%(in M1 generation) and 0.98%(in M2 generation)in gamma ray treatment while it was 2.60%(in M_1 generation) and 0.79% (in M_2 generation) in combination treatment. It was observed that the mutation frequency in M_2 generation was more in combination treatment of 100Gy+0.1% EMS(2.82%) than gamma ray alone (1.09 %) M_2 generation depicts presence of broad chl mutantspectrum, comprising total 8 type mutants. The spectrum of M_2 chl mutants included albino, xantha, chlorina,maculata, Tigrina, auria and viridis are presented in Table 2. In both the treatment, frequency of xantha mutants (in Fig. 2) was maximum (all total) 92.75% (in gamma) and 103.78% (in combined treatment), followed by viridis, albino and yellow viridis in gamma treatment while in gamma+EMS, it was followed by albino, maculata and chlorina. The least frequency of auria type of chl Mutant was recorded 28.14% (in gamma) and 11.11% (in combined treatment).

Albina (Fig. 4 e & g) mutants were completely lack of chl and could survive only a few days. Chlorina (Fig. 4 a,b,c) and yellow viridis (Fig. 4 h & i) had green and yellow green leaves, respectively, are lethal mutations. Aurea (Fig. 3d) had golden yellow coloured leaves and xantha (Fig. 3b) had pale yellow coloured seedlings, tigrina (Fig. 4d) had yellow colour at edges of the leaves furthermore viridis (Fig. 3c) had dark green colour, these type of mutants not only survive but they complete its full lifecycle.

Chl content in chl deficient mutants

The concentration of green pigmentation in the leaves differed among the different types of chl deficient mutants, ranging from chlorina to xantha type of mutants. The chl content (Fig. 5) of various mutants and along with control tissues were examined, the mutants contained significantly less chl than normal plants accept viridis mutant. Among the mutants, albina type was totally devoid of chl while xantha (0.83µg $g^{-1}FW$) and aurea (0.96µg $g^{-1}FW$) contained the least

Table 1. Frequency of chlorophyll mutants induced through gamma and combination treatment (Gamma+EMS) in M_2 generation of *Artemisia annua* L.

Treatments	No. of M_1 plant progeny	M_1 segregates for mutation	M_2 plant scored	M_2 mutants	Mutation frequency (%)	
					M_1 Plants frequency	M_2 plant frequency
Gamma rays (Gy)						
Control	500		1090			
100	500	27	1097	12	5.40	1.09
200	500	21	1070	23	4.20	2.15
300	500	18	921	9	3.60	0.98
Combination treatment (Gy+ %)						
Control	500		1073			
100+0.1%	500	30	1038	28	6.00	2.82
200+0.1%	500	24	994	13	4.80	1.31
300+0.1%	500	13	882	7	2.60	0.79

Table 2. Spectrum and Frequency of chlorophyll mutant in different mutagenic treatments.

Treatment	Total chl mutants in M2 generation	The relative percentage of chlorophyll mutants(%)							
		Albina	Xantha	Chlorina	Tigrina	Maculata	Viridis	Yellow	Auria
Gamma ray (Gy)									
100	12	16.67	33.33	8.33	8.33	0	8.33	16.67	8.33
200	23	13.04	26.09	8.70	13.04	8.70	13.04	8.70	8.70
300	9	11.11	33.33	0	0	0	22.22	11.11	22.22
Gamma+EMS									
100+0.1%	18	11.11	44.44	16.67	0	0	0	16.66	11.11
200+0.1%	13	15.38	30.78	15.38	15.38	0	15.38	15.38	0
3000+0.1%	7	14.29	28.57	0	14.29	28.57	14.29	0	0

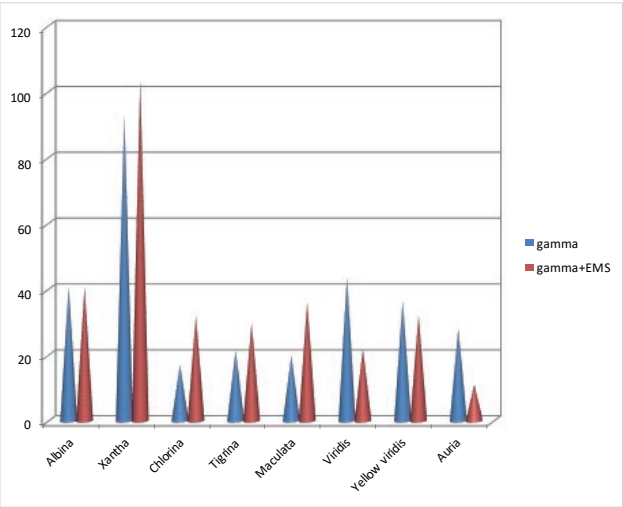

Figure 2. Chlorophyll mutations frequency on mutagen basis in *Artemisia annua* L.

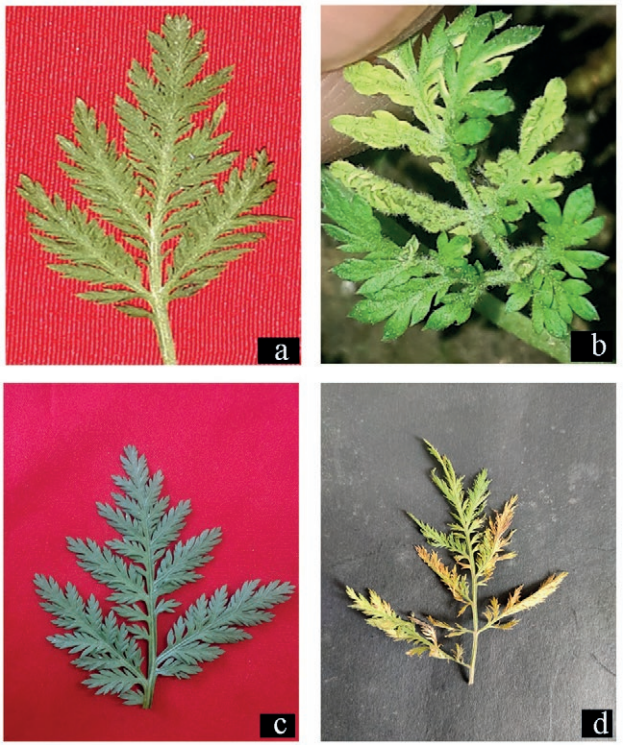

Figure 3. Chlorophyll mutants in *Artemisia annua* L.: a- Control, b-Semi-xantha mutant at seedling stage, c- Viridis mutant, d- aurea mutant

chl and those of viridis (3.86µg g-^{1}FW) the most. The other chl mutants maculata (1.24µg g-^{1}FW), tigrina (1.06µg g-^{1}FW), chlorina (2.46 µg g-^{1}FW) and yellow viridis (3.01µg g-^{1}FW) contained significantly less chl than those of control (3.24 µg g^{-1}FW).

Meiotic study

Meiotic study was done in chl mutant plants which were identified in M_2 generation to screen out genetic disturbance caused by both the mutagen. In control plants 9:9 standard configuration was found. Different types of anomalies were exhibited by treated sets i.e. precocious movement, stickiness, scattering, laggards, bridges, disturb polarity etc. (Fig. 6D–L). Stickiness and multivalent was found to be predominant abnormality in treated sets. Table 3 represented various abnormality frequency and Total Abnormality(TAB) percentage in treated sets. At lower dose of both the treatment TAB% was found to be 6.24±0.15% (Gamma) and 8.93±0.17% (Combined treatment) while at higher dose of both the treatments TAB% was shoot up to 19.71±0.22% (Gamma) and 26.63±0.48% (Combined treatment) . It shows that abnormality percentage is dose dependent.

The results shown in Table 4 indicated that the growth habit of all mutant plants (plant height, inter-

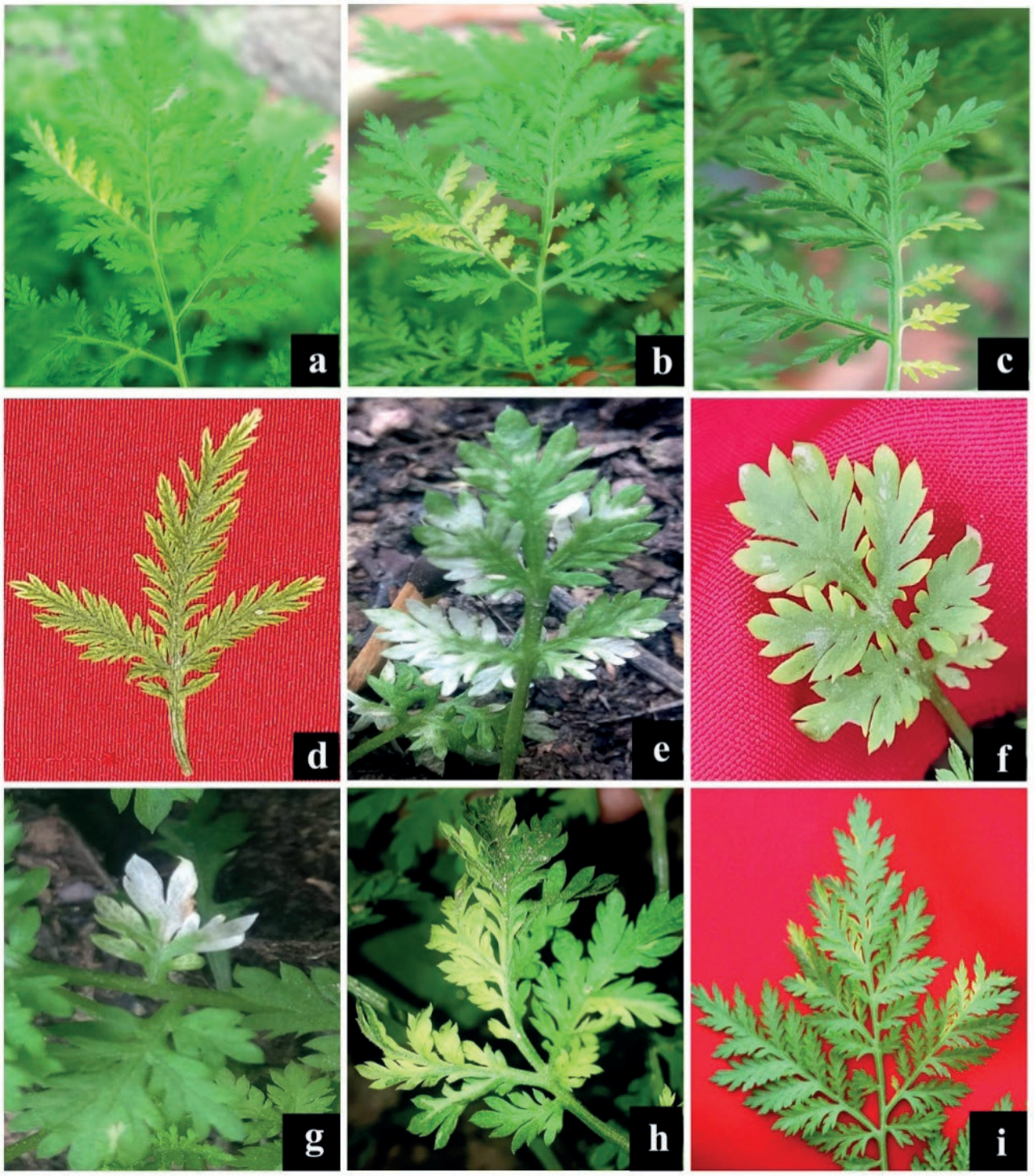

Figure 4. Different types of chlorophyll mutants in *Artemisia annua* L. a. b. c. Chlorina mutant, d. Tigrina mutant, e. g.. Albina mutant, f. maculate mutant, h. i. Yellow viridis mutant

nodal length, leaf area, No. of primary branches, days to 50% flowering and Days to maturity) were different than those of the control plants. As height (cm) of the control plant was observed 103.20±0.88 although at 100Gy and 100±0.1% it was increased i.e. 112.30±1.01and 110.30±1.09 which decreased as the

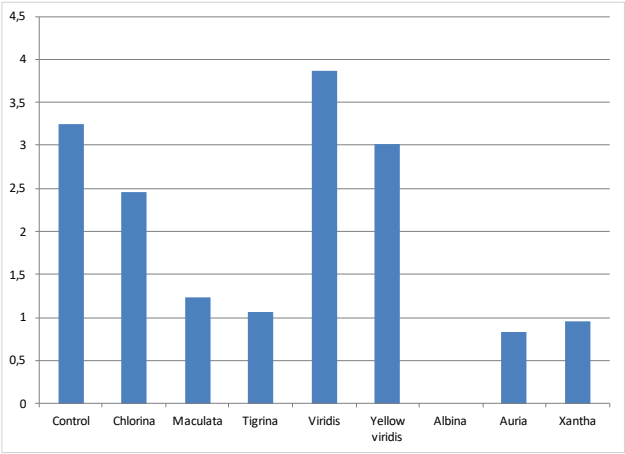

Figure 5. Chlorophyll content in normal type and chlorophyll deficient mutants of *Artemisia annua* L.

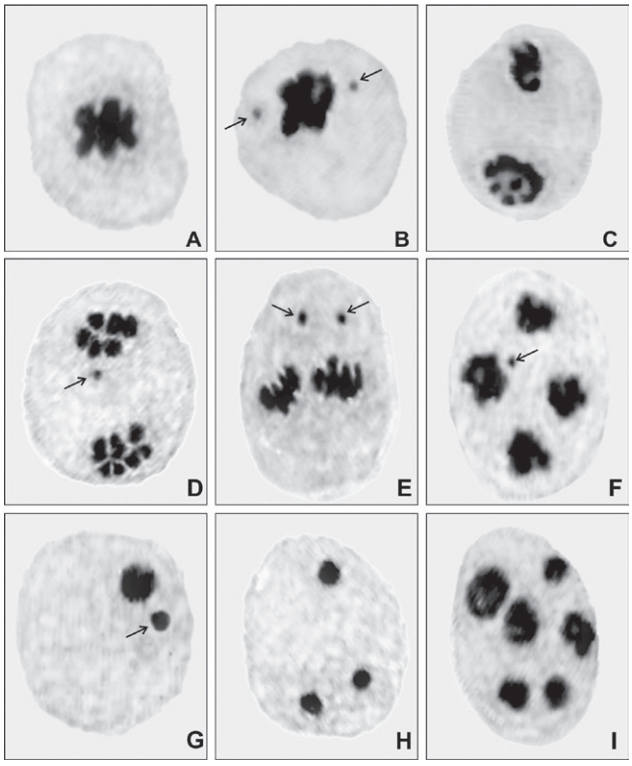

Figure 6. Different types of abnormalities in treated plants of *Artemisia annua* L.: **A.** Stickiness at metaphase I, **B.** Two precocious chromosome at Sticky metaphase I, **C.** Stickiness at unoriented Anaphase I, **D.** One laggard chromosome at Anaphase I**, E.** Two precocious chromosome at Metaphase II, **F.** One laggard chromosome at Anaphase II**, G**. Mononucleate condiotion, **H.** Binucleate condition, **I.** Multipolarity(Scale bar= 10.13μm)

Figure 7. Some phenotypic variants in *Artemisia annua* L. **A**-Prostrate variant, **B**- Dwarf variant **C**- Tall variant.

doses of mutagen increases. Internodal length, leaf area and primary number of branches were improved at the lower dose of gamma rays but as the doses increases all these character significantly minimally decreased. At lower dose of gamma and gamma+EMS plant flowers earlier and matures faster as compared to control. Control plants were flower in 56 days and mature in about 155-156 days. While At 100Gy and 100+0.1%, days to 50% flowering was observed 50.00±0.57 and 52.00±1.03 furthermore days to maturity was 145.00±4.50 and 143.00±1.00. Fig. 7 shows the dwarf variant and tall variant. Dwarf variant was found at 300+0.1% while tall variant screen out at 100Gy dose and also some plant become prostrate as shown in Fig. 7A.

Table 3. A comparative account of Chromosomal anomalies in induced through Gamma ray and combination (gamma+EMS) treatment in *Artemisia annua* L.

Doses (Gy)	No. of PMC's observed	Metaphasic Abnormalities (%) (Mean±S.E.)							Anaphasic Abnormalities (%) (Mean±S.E.)					Oth. (%)	T.Ab. (%)	Pollen fertility (%)
		Sc	Pm	St	Un	Mv	Sa	Br	Lg	Un	St	Asy	Dp			
Control	330	-	-	-	-	-	-	-	-	-	-	-	-	-	-	94.41±1.46[a]
100	310	0.43±0.10	1.08±0.10	0.65±0.19	0.43±0.11	0.43±0.11	0.32±0.19	0.54±0.10	0	0	0.89±0.10	0.65±0.01	0.32±0.04	0.53±0.10	6.24±0.15	92.37±1.85[a]
200	290	0.80±0.30	1.25±0.28	0.92±0.13	1.14±0.21	0.93±0.25	0.56±0.12	0.81±0.13	0.80±0.19	1.04±0.02	0.69±0.01	0.93±0.25	0.69±0.01	0.58±0.12	11.25±0.14	87.19±1.87[b]
300	291	1.84±0.12	2.19±0.27	1.26±0.09	1.95±0.09	1.37±0.18	1.27±0.14	1.38±0.21	1.37±0.10	1.62±0.34	1.72±0.18	1.14±0.08	1.37±0.16	1.25±0.29	19.71±0.22	70.25±1.59[c]
Control	358	-	-	-	-	-	-	-	-	-	-	-	-	-	-	95.22±0.58[a]
100+0.1%	385	0.61±0.10	0.86±0.07	1.31±0.19	0.69±0.07	0.51±0.14	0.51±0.14	0.61±0.10	0.43±0.22	0.60±0.07	0.79±0.17	0.78±0.16	0.61±0.09	0.61±0.09	8.93±0.17	85.67±1.15[a]
200+0.1%	370	1.44±0.09	1.44±0.23	1.29±0.66	1.53±0.23	1.08±0.14	1.08±0.04	1.17±0.10	0.90±0.06	1.28±0.28	1.35±0.14	1.46±0.29	1.07±0.12	1.43±0.15	16.54±0.85	77.12±1.73[b]
300+0.1%	362	1.39±0.32	1.66±0.19	3.96±0.38	1.66±0.04	2.67±0.19	1.57±0.10	1.56±0.33	2.41±0.38	1.95±0.32	1.11±0.18	2.94±0.18	1.74±0.20	2.01±0.47	26.63±0.48	61.29±0.85[c]

Abbreviations: S.E.- Standard Error;**Sc**- Scattering; **Pm**- Precocious movement; **St**- Stickiness; **Un**- Unorientaion; **Mv**- Multivalent; **Sa**- Secondary associations; **Asy**- Asynchronous; **Br**- Bridge; **Lg**- Laggard; **Dp**- Disturbed polarity; **Oth**- Others; **T.Ab.** –Total abnormalities, Means are followed by lowercase letter is statistically significant at $p < 0.05$.

Table 4. Gamma ray and Combination treatment (Gamma+EMS) on some qualitative and quantitative traits of chlorophyll mutants seedling in *Artemisia annua* L. (M2 generation).

Morphological traits	Control Mean±S.E.	Chlorophyll mutant lines					
		100 Gy Mean±S.E.	200Gy Mean±S.E.	300Gy Mean±S.E.	100±0.1% Mean±S.E.	200±0.1% Mean±S.E.	300±0.1% Mean±S.E.
Plant height (cm)	103.20±0.88[b]	112.30±1.01[a]	108.22±1.60[b]	86.30±1.48[c]	110.30±1.09[a]	88.30±1.44[b]	52.50±1.30[c]
Internodal length (cm)	7.50±0.09[ab]	8.60±0.13[a]	7.40±0.15[b]	6.90±0.13[c]	8.31±0.11[a]	6.30±0.15[b]	5.60±0.14[c]
Leaf area (per m^2)	35.60±0.48[b]	38.20±0.47[a]	28.50±0.46[c]	27.80±0.42[c]	34.20±0.42[b]	26.60±0.49[b]	22.30±0.43[c]
No. of primary branches	25.00±0.86[b]	30.00±1.08[a]	27.00±0.86[b]	26±1.06[a]	21±0.83[ab]	19±0.76[b]	17±0.63[c]
Days to 50% flowering	56.00±0.43[b]	50.00±0.57[c]	55.00±0.60[b]	66.00±2.16[a]	52.00±1.03[b]	65.00±2.60[a]	79.00±2.79[a]
Days to maturity	155.00±1.06[b]	145.00±4.50[c]	150.00±4.01[ab]	170.00±3.55[a]	143.00±1.00[c]	172.00±3.05[a]	159.00±3.68[b]

Abbreviations: S.E.- Standard Error, Means are followed by lowercase letter is statistically significant at $p < 0.05$

DISCUSSION

In mutation breeding programs, the selection of an effective and efficient mutagen concentration and growth condition is essential to produce a high frequency of desirable mutations(Arisha et al. 2014). Chl mutation frequency in M_2 generation is one of the most dependable measures for evaluating the mutagen-induced genetic alternations. The spectrum of chl mutations was found to be dependent on the genetic background of the genotype. Moreover chl mutation frequency increased with the increase in dose of gamma rays both individually as well as in combination with EMS in all the varieties.

In the present investigation the germination percentage and plant survival were reduced significantly. The reduction in germination may be due to the seeds engrossing the mutagen, which subsequently reaches the meristematic regionof seeds and affects the germ cell (Serrat et al. 2014). Also, a reduction in germination may be because of the damage of cell constituents (Kumar et al. 2013), alteration of enzyme activity or delay or inhibition of physiological and biological processes (Talebi et al. 2012).Reduction in plant survival in treated population may occur due to various factors such as cytogenetic damage and physiological disturbances (Sato and Gaul 1967)and disturbances in balance between inhibitors of growth regulators and promoters (Meherchandani 1975).

Ionizing radiation singly not produces much chl mutation as combination treatment produces. Among the chemical mutagens, EMS is now being widely accepted as the most efficient and influential mutagen which induces highfrequency and wide spectrum of mutation. When EMS combined with radiation it not only causes synergistic effect but affect mutation in a specific ways. Singh (et al. 1999) reported that combined treatments of gamma rays and EMSwere most effective in producing chl mutation frequency than their individual treatments in *Vigna* Chl mutationsinduced by EMS, gamma rays and other mutagens applied individually or in combination were reported by a Kumar (et al. 2009) and Gandhi (et al. 2014) in *Vigna radiate,* Bolbhat and Dhumal (2009) and Kulkarni and Mogle (2013) in *Macrotyloma uniflorum* (Lam.) Verdc.*,* Sharma et al. (2010) in *Pisum sativum* L. Gaur et al. (2013) in *Capsicum annuum.* Lower doses of gamma and gamma+EMS mutation frequency increases but it significantly decreased at the higher doses. Sharma (1970) reported that chl mutation frequency decreased at higher doses when calculated on segregating M1 familiesbasis. For both the treatment higher frequency of chl mutation with moderate doses of mutagens was observed. It seems that the strong mutagens reach their saturation point even at lower or moderate doses in the highly mutable genotype. With increase in dose further than a limit, the strong mutagens become more toxic than the higher doses of relatively weaker mutagens and do not increase mutation frequency (Kolar et al. 2011). Moreover mutation frequency observed maximum in gamma+EMS treatment both in M_1 and M_2 generation in comparison to gamma. It may be due to EMS, a chemical mutagen which causes formation of new sites for mutation. So use of gamma followed by EMS suggests that the chemical mutagen is more efficient in inducing mutations of genes needed for chl development (Shah et al. 2006).

In both the treatment, highest frequency of xantha mutant was observed, The highest frequency of xantha mutants ismay be due to the genes for xanthophylls development that are readily accessible for mutagenic action (Similar reports were already given by Lal (et al. 2009), Khan (et al. 2005), Haq (1990) . These mutants could not survive more due to block in chl synthesis (Blixt 1961). In gamma, after xantha viridis was the second highest mutant, these mutant survived tillmaturity. Viridis attributed may be due involvement of polygene genes for the formation of chl. In combinedtreatment second highest mutant recorded was albino, these type of mutant formed may be due to deficiency ordegradation of chl formation enzyme. in chickpea all chl mutants including albina type were in general morefrequent in EMS treatments than in gamma rays (Singh 1988).

Chl is a vital biomolecule which plays a critical role in the life processes of allplants. Plants photosynthesis by absorbing light and transferring light energy to the reaction centers (chl molecule)of the photosynthetic system. Thus, Chl is essential for plant development and agricultural production (Eckhardt et al. 2004; Flood et al. 2011). Chl development seems to be controlled by many genes that are located on different chromosomal sites (Wang et al. 2013). It derivesby the formation of a long chain of biochemical process in which lots of loci were involved. The phenotypes of leaf color mutations are varied and are affected by different genetic and environment factors. Mutant plants leaf shows lower or higher chl content than normal leaf. This revealed that rate of change in the content of Chl *a* and Chl *b* was not the same among the mutants, possibly was due to the impair of Chl *b* synthesis during chloroplast development (Kolar et al. 2011). Ionizing radiation and chemical mutagen at higher concentration affectschloroplast thylakoid membrane which causes disability in chl manufacturing. This chl deficiency reduced the rateof plant growth. Mutant plants with a higher (Nielsen et al. 1979) like viridis or a lower like chlorine, xantha (Vaughn et al. 1978; Wu et al. 2007) Chl *a*/*b* ratiothan that of their

respective normal plants have been reported to be able to survive photoautotrophically. Mutations affecting the production of chl are important for identifying gene function and the elucidation of chl metabolism and its regulation (Wu et al. 2007).

Cytological investigation defines the specific responses of different genotypes to a specific mutagen and it is also provides significant evidences for the selection of desirable traits (Kirchhoff et al. 1989).In the present appraisal chl mutant depicts various anomalies such as , stickiness, multivalent, precocious movement of chromosome, laggard and bridges. Stickiness could arise due to depolvmerization of' nucleic acid caused by mutagenic treatment (Avijeet et al. 2011). The formation of multivalent (Fig. 6 G) may also be attributed to the abnormal pairing and non-disjunction of bivalents (Jabee et al. 2008). Jafri (et al.2011) suggested that precocious movement (Fig. 6 D&I) of chromosomes was probably caused by spindle dysfunction. Laggard formation (Fig. 6F,K &L) is due to delayed terminalisation, chromosomal stickiness or failure of chromosomal movement (Reddy and Munirajappa 2012). Due to direct action of mutagen target proteins gets defective and creates the disturbance during chromosome separation and it forms bridges (Kumar and Gupta 2009). The increment in the chromosomal aberrations might perhaps be due to the interactions of ionizing particles with the protoplasm, mediated through the excitation introduced by radiation that ultimately has increased the aberration frequency (Shukla nee Tripathi and Kumar 2010).

When mutagens affects plant tissues internally it get accommodated and damage the cell or genes whichphenotypically can be seen. Genetically, during the M1 generation the probability of the occurrence of phenotypicmutation is extremely low and only dominant mutations can be identified (Roychowdhury and Tah 2013). During the M2 generation, the chancefor identifying visible changes or phenotypic mutations should be higher and mostly due to genetics. Thereforeobserved mutations in the M2 generation are considered more stable (Parry et al. 2009). The plant height at lower doses of gamma and gamma+EMS was significantly increased but higher doses of both the treatment causes inhibition /depression in plant growth. As according to Van Harten (1998) said, at high amount irradiation could cause the physiological damages such as inhibit cell division, death of cell and growth rate and genetic changes on the plants by producing free electrons radical. Internodal length, leaf area and primary number of branches were increased at 100Gy dose of gamma. These characters were significantly higher at lower concentration but at higher concentration it shows a reduction pattern. Treatment of gamma rays and EMS exhibited increase in mean values of number of primary branches in *Lathyrus sativus* (Waghmare and Mehra (2000). In mutation breeding programme, yield and its attributed traits are very important parameters because ultimately breeders want to improve yield and related characters (Shahwar et al. 2020). Similar result was givenby Hanafiah (et al. 2010) in irradiated *Glycine max* (L.) Merr., var. Argomulto seeds with gamma rays showsphenotypic variations that occur on M1 plants which affects plant growth development and production and also was given by Sinuraya (et al.2017) in *Allium cepa* assay. Thilagavathi and Mullainathan (2011)concluded that the decrease in quantitative traits have been attributed to the physiological disturbance or chromosomal damage caused to the cellsof the plant by the mutagen as Williams (et al.1990) observed that due to nucleotide substitutions and insertion or deletions polymorphism occurred between individuals. In comparison to control treated plant at lower dose (100Gy,200Gy, 100+0.1%) flowers early and matures more rapidly. At 200Gy+0.1% dwarf mutant with early maturing plant was identified. Panigrahi (et al.2015) suggested that significant variations in quantitative parameters may showstable gene mutations in the next generations. Konzak (et al.1969) in wheat and Shakoor (et al. 1978) in *Triticale* reported that polygenes are responsible for semi dwarf character. Qin (et al.2008) reported dwarf rice mutants caused by single gene. Increased height and number of branches were due to loss of apical dominance which leads to lateral transport of growth hormone which results increased number of branches and bushy appearance as also observed earlier in *Vicia faba* (Shahwar et al. 2017), lentil (Solanki et al. 2004). Physical mutagen causes random change in the growth regulatory genes of plants but chemical mutagen exactly targets their mutagenic site through point mutation. This is the reason combination treatment proved to be more mutagenic and produces good amount of mutants. The selection of effective and efficient mutagens is most important to recover the spectrum while high frequency of desirable mutations and efficiency of a mutagen indicates relatively less biological damage in relation to induced mutation (Solanki and Sharma 1994).

CONCLUSION

This investigation revealed the potency of gamma and in combination with EMS ,on increasing genetic diversity and demonstrated the successful program of induced mutagenesis in the *Artemisia annua*L. In this breeding programme a total of 44 mutant in gamma and

67 mutants in combination treatment were segregated. Various chl variants were identified in treated sets. Xantha was predominant among all the variants as most of the leaves found pale yellow colour. It is noticed that these changes are differentially sensitive to gamma and gamma+EMS and the appearance of new mutants would very helpful in maintaining the genetic purity of plant variety. So it should be important to identify desirable mutant plants through isolation and selection method. The cytological analysis of these mutants showed that these changes were induced due to changes in chromosome number, structure, base substitution and deletion. For *Artemisia* LD_{50} recorded as 200Gy. 100Gy and 100+0.1%EMS was noticed good for plants growth and development as plant height, internodal length, leaf area and primary number of branches improved. So gamma and gamma+EMS induced reasonable chl mutations, hence all these treatments could be used in mutation breeding programs for inducing viable mutations only threshold dose of mutagen should be identified.

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