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ORCID
SP: 0000-0003-0653-6167
GK: 0009-0007-3597-9289
NKT: 0009-0000-5848-1319
JY: 0009-0001-8357-9585

Gamma Irradiation Effects on *Salvia hispanica* L. seeds in M2 Generation: A comprehensive study of genetic variation and phytochemical responses

Satya Pandey*, Girjesh Kumar, Naveen Kumar Tiwari, Jyoti Yadav

Department of Botany, University of Allahabad, UP-211002 (India) Prayagraj, India
*Corresponding author. E-mail: satyap.sp90@gmail.com

Abstract. Gamma irradiation is a powerful tool in mutation breeding, promising to boost plant productivity and yield while influencing phytochemical composition and morphological traits. This study focuses on understanding the effects of gamma irradiation on Chia (*Salvia hispanica* L.) seed development, encompassing germination, growth, and photochemical properties. Ionizing radiation has proven to be a potent physical agent in mutation breeding initiatives, potentially enhancing plant productivity and yield. A comprehensive analysis was conducted, encompassing the application of distinct gamma irradiation doses ranging from (0, 50,100, 150, 200, and 250 Gy) in M_1(2021-22) and M_2 (2022-23) and oils were extracted in M_2 generation using the Soxhlet technique. Various parameters, including sterol composition, fatty acid composition, tocopherol content, and fatty acid value (FAV), were meticulously analyzed using the Gas Chromatography-Mass Spectrometry (GC-MS) technique. Increased phytochemical viz., Alpha-linolenic acid (60.23%), Linoleic acid methyl ester (19.78%), and Palmitic acid (11.96%) were obtained at 100 Gy irradiation that had not been reported in earlier research. Therefore, the potential of gamma irradiation to enhance chia seeds' nutritional and phytochemical properties exists. This insight holds promise for advancing seed development and overall plant performance, offering valuable prospects for crop improvement and the creation of nutrient-rich agricultural products.

Keywords: *Salvia hispanica* L., Gamma irradiation, GC-MS analysis, Ionizing radiation, Alpha-linolenic acid.

1. INTRODUCTION

In recent years, there has been a notable surge in the utilization of chia seeds within the food, dietary supplement, and cosmetic industries. This surge in popularity can be attributed not only to the seed's valuable chemical composition and biological activity but also to its widespread availability. Chia (*Salvia hispanica* L.), commonly known as Mexican chia or Spanish sage and a member of the Lamiaceae family, is grown in tropical and subtropical countries (Ixtaina et al., 2008). Chia seeds are frequently used in the functional food industry due to their rich composition. They typically contain approximately 30–33% lipids, 15–25% proteins, and 26–41% carbohydrates, along with various vitamins, essential minerals, and a substantial dietary fiber content ranging from 18–30% (Ullah et al., 2016). Furthermore, chia seeds contain a wide range of polyphenols, known for their antioxidant properties and potential health benefits (Ixtaina et al., 2011). This is due to their rich omega-3 fatty acid content and a favorable omega-3 to omega-6 fatty acid ratio popular among individuals adhering to plant-based diets, including vegetarians and vegans (Sebastiani et al., 2019).

Mutation breeding techniques are being employed to introduce genetic diversity into chia due to its limited genetic base. In recent years, they have gained widespread use, particularly for enhancing the genetic diversity of vegetatively propagated crop plants. Gamma irradiation is a form of ionizing radiation widely used in various scientific disciplines, including agriculture and plant breeding. Its ability to induce genetic variation and stimulate physiological responses in living organisms makes it a valuable tool (Ali *et al*., 2015). In our experimental design, we employed triplicate treatments, subjecting chia seeds to gamma irradiation, while a control group was closely monitored for changes in morphology, cytology, and phytochemical responses.

2. MATERIAL AND METHOD

2.1 Seed procurement

Inbred seeds of the chia plant were obtained from NutriPlanet Private Limited, Bengaluru-520068, Karnataka, India. Two varieties of chia plants, namely Black and White, were provided. Given the heightened economic significance of black seeds and their associated properties, these were selected as the focal variety for this study.

2.2 Seed irradiation treatment

Inbred chia seeds were enclosed within individual pockets and subjected to distinct irradiation doses (50, 100, 150, 200, and 250 Gy) using a Cobalt-60 source at NBRI, Lucknow. The irradiation process was carried out using gamma rays at a dose rate of 7.247 kGy.

2.3 Seed sowing

After treatment, all the irradiated seeds were sown in the triplicate set of pots following a randomized complete block design (RCBD) in the field. The temperature and humidity were recorded at 25±2 °C and 76% respectively.

2.4 Morphological traits

Seven days after seed sowing the germination percentage of the plants was calculated and plant survival rates were recorded after 30 days. Plant height was gauged at 45-day intervals, accompanied by the observation of various leaf mutants arising from diverse exposures. The onset of plant flowering, occurring around day 120, was scrutinized, encompassing an examination of variations in their inflorescence patterns.

2.5Meiotic study- To facilitate the cytological observations, the young floral buds of plants were fixed in carnoy's fixative (Alcohol 3: Glacial Acetic Acid 1) for 24 hours. These buds were subsequently preserved in 90% alcohol. Taken small size anther and gently teased using needle and forceps. Staining was accomplished using a 2% acetocarmine solution. Microscopic observations were carried out using a Nikon phase-contrast microscope (Nikon Eclipse E200, Japan). The identification of cytological abnormalities was undertaken and the total abnormality percentage (%) within the treated sets was calculated.

2.6 Extract extraction

For GC-MS studies, harvested mature seeds from the M_2 generation subjected to gamma treatment were utilized, with corresponding control sets. Methanolic extraction was prepared by placing 10 grams of seeds in 250 ml of methanol solvent within a Soxhlet apparatus for oil extraction. Filtered samples were stored in an Eppendorf tube and labeled with different irradiation doses.

2.7 GC-MS Analysis

Methanolic extracts were prepared for both gamma-treated and control seeds separately by adding 10gram seeds to 250 ml of methanol transferring them to the Soxhlet apparatus and extracting the essential oils out of it. Extracts were further filtered by using Whatman filter paper. Model GCMS-QP2010 serial no. 0205251 SHIMADZU was used for GC-MS analysis. After filtration 6 µl of Methanolic extract was injected into the column and analyzed. The conditions were set as under Injection temp: 260 °C, column oven temperature 100°, injection mode split, total flow -16.3 mL/min, Pressure -90.5

kPa, Ion Source Temp. 220 °C, Interface Temp. -270 °C, Solvent ut time: 3.50 min, Detector gain mode: relative, Relative Detector Gain: +0.00 kV, Threshold: 1000. The chemical composition was elucidated, encompassing saturated and unsaturated fatty acids, sterols, steroids, vitamins, and other metabolites. Identification of different metabolites was based on their fatty acid content, characterized by area percentage and retention time.

2.8 Statistical analysis

Observed data underwent analysis utilizing SPSS 16.0 software. A one-way analysis of variance (ANOVA) was conducted, followed by Duncan's Multiple Range Test (DMRT, with significance at $P < 0.05$) for mean separation. For Graphical representations using Sigma Plot 10.0 software. Actual means and standard errors were computed and the dataset was subjected to further analysis of variance.

3. RESULTS AND DISCUSSION

3.1 Germination and survival rates

After 30 days differences in parameters were observed in M_1 generation. Various morphological parameters such as germination rate, survival rate, plant height, and inflorescence axis were meticulously recorded. Comparative data of germination and survival of both the M_1 (90.38±1.49% at 50 Gy to 73.45±2.85% at 100 Gy) and M_2 (92.16±1.74% at 50 Gy to 75.58±2.83 100 Gy) generations are shown in (Fig. 1). Control germination was recorded 95.36±0.84%. In all treatment sets the germination percentage of M_2 were higher compared to M_1. This demonstrates a dose-dependent response in chia plant germination to gamma irradiation. This result is consistent with (Hanafy and Akladious 2018) regarding the negative impact of high doses of gamma rays on plant morphology and growth. A similar finding was reported by (Aparna *et al*., 2013 in *Arachis hypogaea* L.).

Survival rates exhibited a negative correlation with increasing irradiation doses, with the control group showing the highest survival rate (93.56± 0.1.02). In treatment sets at 50 recorded 85.23±1.41 to the lowest survival rate observed at 250 Gy irradiation (65.24 ± 2.77) shown M_1 generation and increased from 88.65±1.15% at 50 Gy to 69.35±2.54% at 100 Gy in M_2 generation. These findings align with the notion that ionizing irradiation can have adverse effects on various plant traits, including germination and survival (Mittler, R. 2002). Similar trends were observed in *Cuminum cyminum* seedlings by (Verma *et al*. 2017).

3.2 Morphological traits

Plant height increased significantly at 100 Gy (75.48 ± 1.68 cm) compared to the control (72.46±0.98 cm) in the M_1 generation. (Fig. 2A) In the M_2 generation plant height increased prominently as compared to M_1 recorded with bushy mutants recorded at lower doses of exposure as shown in (Fig. 3 L). Inflorescence axis length also increased at 100 Gy (12.42±0.21 cm) M_1 and (13.25±0.24 cm) enhanced in M_2 but declined with higher radiation doses (Fig. 2B). Different types of leaf mutants were characterized, including color, shape, and size variations.

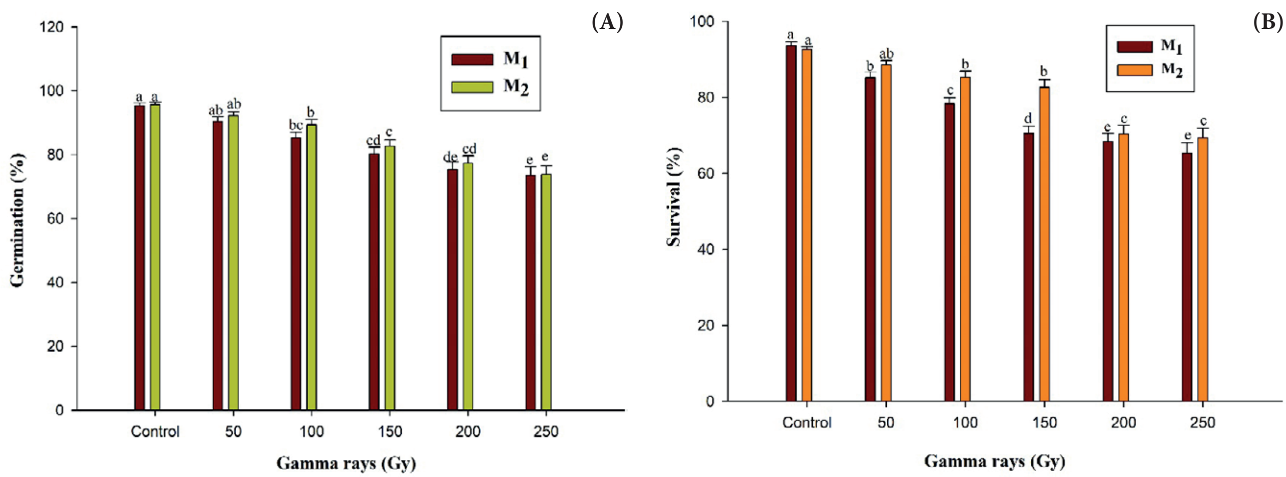

Figure 1. The morphological parameters Germination percentage (A) and Survival percentage (B) of the M1 and M2 generation about gamma radiosensitivity were investigated through seed treatment at $P < 0.05$ significance enhancement as in ANOVA .

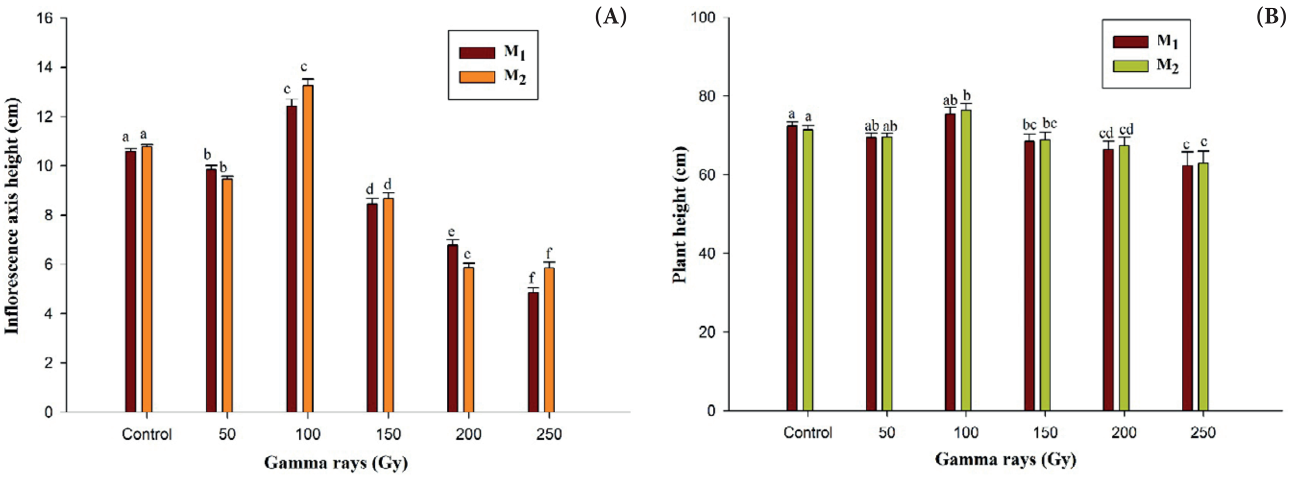

Figure 2. Graph representing morphological observations of plant height in M1 and M2 generation (A) and inflorescence axis in M1 and M2 generation (B) difference at $P < 0.05$ significance enhancement as in ANOVA .

Various leaf mutants were observed in the M_2 generation, including Semi-xantha mutants, Albo-viridis, Semi Albina, Yellow-viridis, Maculata mutants, Tricotyledonous leaves, Bifurcated leaves and single axes with three inflorescences in M_2 generation depicted in (Fig. 3). The hypothesis proposed by Wi *et al*. (2007) suggests that lower doses of gamma irradiation may induce growth by influencing hormonal activities and bolstering antioxidant defenses in plant cells. This could enable plants to better withstand daily stress factors. The underlying reasons for these chlorophyll mutants and the genes and proteins involved remain subjects of ongoing research, as noted by Ahumada-Flores *et al.* (2020). Several authors have previously reported different types of chlorophyll mutations, such as Xantha, Albina, Viridis, and Chlorine, among others (Kolar *et al*., 2011; Arisha *et al*., 2015; Verma *et al*., 2018).

A novel observation was made regarding tricotyledonary true leaves at a specific node in *Salvia hispanica L.* plants (Fig 3D). Similarly, tricotyledonary seedlings have been reported in sunflowers by (Hu *et al*. 2006), who suggested that this phenotype is controlled by a few recessive genes, which are typically masked by dominant traits but occasionally manifest due to the lethality of masking genotypes. These tricotyledonary seedlings bear three true leaves at each internode. In the case of Kalmegh, Dwivedi *et al*. (2021) also reported the presence of trimeric true leaves in the M_2 generation. It has been observed that lower-dose gamma irradiation has a stimulatory effect and enhances various traits, consistent with findings by Kim *et al*. (2001). However, it should be noted that higher doses of gamma radiation beyond 100 Gy had detrimental effects, consistent with the findings of Hanafy and Akladious (2015), who explained that the highest gamma-ray dosage negatively impacted fenugreek morphology and growth when compared to control plants. Seed weight increased in the treatment group compared to the control, with the most significant change observed at 100 Gy (1.76±0.02 g of 250 seeds) compared to the control (1.32±0.031 g of 250 seeds) in Fig. 4B. Seed weight (in 1 cm square) increased, as depicted in (Fig. 4B)**.**

3.3 Cytological abnormalities

Meiotic studies of pollen mother cells (PMCs) revealed various cytological abnormalities, including scattering, stickiness, laggard movement, and bridge formation depicted in (Fig. 5). The percentage of abnormal PMCs (Tab %) increased with higher doses of gamma irradiation, ranging from (4.62 ± 0.14 to 12.59 ± 0.31) in Table 1. Pollen sterility also increased with irradiation dose, with the control group showing the highest fertility rate (97.47 ± 0.99 %) compared to the lowest at 64.96 ± 2.48 % mentioned in Table 1. The inhibitory effect on the cell cycle of gamma irradiation at higher doses has also been reported earlier in *Allium cepa* by (Ahirwar, 2015). Furthermore, (Kumar and Dwivedi *et al*. 2021) have reported that bridge formation can result from spindle dysfunction induced by higher-dose mutations. (Kumar and Gupta 2009) suggested that gene mutations or the direct action of mutagens on target proteins responsible for chiasma terminalization during diakinesis at meiosis-I can lead to structural defects in these proteins. These defects ultimately impair their proper functioning, resulting in the formation of chromosomal

Figure 3. Leaf mutants after gamma irradiation observed in M_2 generation **A.** control; **B.** Semi-xanthan**; C.** Alboviridis; **D**. Tricotyledonous leaf; **E.** Bifurcated; **F** single axis with three inflorescence bud; **G.** Semi-albina; **H.** Yellow-Viridis; **I.** Xantha mutant; **J.** Maculata; **K.** plant height variation in M2 generation: control group with treatment sets in M_2 generation; **L.** control with Bushy mutant; **M.** Control Inflorescence; **N.** inflorescence with fused axis; **O.** Seed size in treatment and control.

bridges. Stickiness, for example, may result from imbalances in spindle fibers caused by mutagenic treatment. (Jabee *et al.,* 2008).

Furthermore, the study noted a decline in pollen fertility due to the formation of sterile pollen as a side effect of mutagenic treatment. This increase in pollen sterility with higher irradiation doses poses a risk to the survival of plant genotypes. A decline in pollen fertility was attributed to the formation of sterile pollen, primarily resulting from the adverse effects of mutagens on the male reproductive organs. It was observed that the rate of pollen sterility increased with escalating doses of irradiation, ultimately leading to the production of non-via-

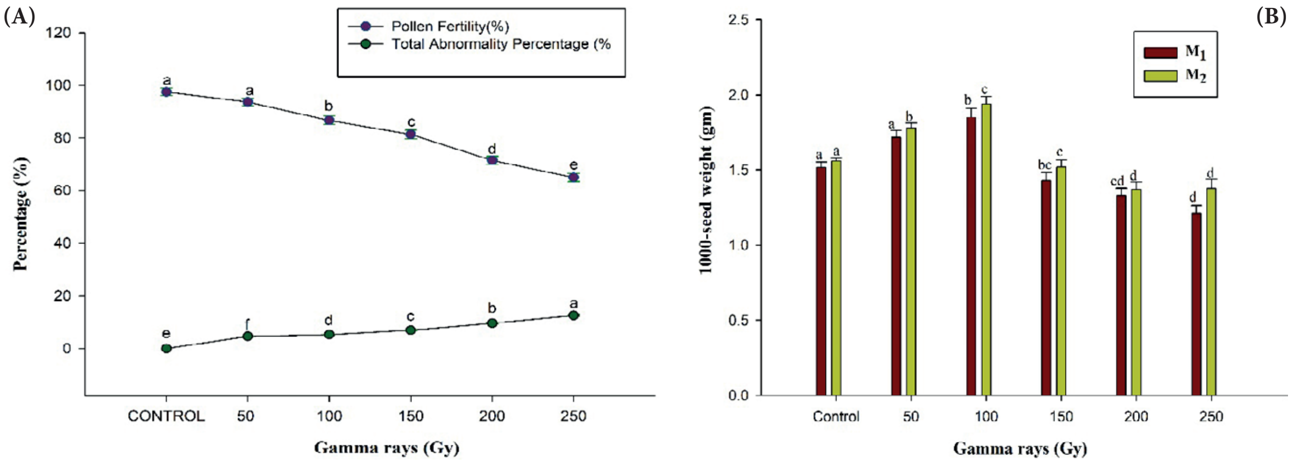

Figure 4. Graph (A) shows a negative correlation between Pollen fertility and Total Abnormality Percentage (%) with increased doses of gamma irradiation and (B) represents seed weight difference in M1 and M2 generation .

Table 1. Gamma irradiation-induced cytological abnormalities and their percentage in *Salvia hispanica* L.(2n=12) during Meiosis.

Treatment	PMC	METAPHASIC ABNORMALITY			ANAPHASIC ABNORMALITY				OTH	TAB	POLLEN FERTILITY
		SC	ST	PM	AST	ASC	AUN	BG			
CONTROL	458	-	-	-	-	-	-	-	-		97.47±0.99
50 Gy	441	0.98±0.09	0.53±0.07	0.61±0.08	0.61±0.15	0.61±0.07	0.46±0.01	0.22±0.01	0.00±0.00	4.62±0.14	93.65±0.59
100 Gy	443	0.97±0.06	0.83±0.07	0.67±0.22	0.60±0.15	0.53±0.07	0.67±0.12	0.22±0.13	0.22±0.13	5.26±0.39	86.67±2.97
150 Gy	393	1.44±0.09	0.93±0.09	0.93±0.30	0.85±0.08	1.19±0.09	1.23±0.16	0.26±0.15	0.25±0.15	6.95±0.21	81.36±2.32
200 Gy	379	1.85±0.14	1.14±0.18	1.41±0.10	1.32±0.16	1.32±0.16	1.23±0.16	0.35±±0.09	0.36±0.24	9.57±0.71	71.49±2.13
250 Gy	354	1.98±0.17	1.50±0.07	1.79±0.27	1.60±0.25	1.78±0.07	1.60±0.20	1.02±0.08	0.61±0.23	12.59±0.31	64.96±2.48

Where, PMC's- Pollen mother cells, Sc- Scattering of chromosomes, Pm- Precocious movement of chromosomes, St- Stickiness of chromosomes, Ast- Anaphasic stickiness, Aun- Anaphase Unorientation, Oth- Others, Tab- Total abnormality percentage (p= <0.5).

ble pollen. This phenomenon poses a potential threat to the survival of plant genotypes within the system. Singh and Kumar (2020) reported a similar pattern in *Artemisia annua*, observing an increase in pollen sterility proportional to the irradiation dose. (Jagtap and More 2014) conducted an analysis for *Lablab purposes* and arrived at a similar conclusion: plant sterility intensifies as the dose of physical or chemical mutagens increases.

3.4 Biochemical composition

Gamma irradiation led to alterations in the fatty acid composition of chia seed oil in the treatment and control set analyzed in M_2 generation Table 2. Saturated fatty acids, such as Palmitic acid (16:0), showed an increase at 100 Gy (8.42%) compared to the control (7.52%). Unsaturated fatty acids, including Alpha-linolenic acid, Linolenic acid methyl ester, and Alpha-Monosterin, exhibited enhancements at various irradiation doses depicted in Table 2Vitamin E was the predominant vitamin observed, with a significant increase of 0.36% following gamma irradiation at 100 Gy. Additionally, gamma-sitosterol, a sterol compound, showed a notable increase from 0.06% in the control group to 9.89% in the treated samples. Other sterols exhibited variable responses to the irradiation treatment. The presence of fumaric acid, triterpenoids, and corticosteroids was also detected and showed alterations with gamma irradiation. The metabolites were categorized into five main groups: saturated fatty acids, unsaturated fatty acids, esters, vitamins, and stigmasterol with their area percentages. The GC-MS analysis of gamma-treated seeds of M_2 has revealed noteworthy changes, including a significant enhancement in the content of unsaturated fatty acids. Significant increases were observed in various phytochemicals of chia seeds following gamma irradiation at 100 Gy, including Alpha-linolenic acid

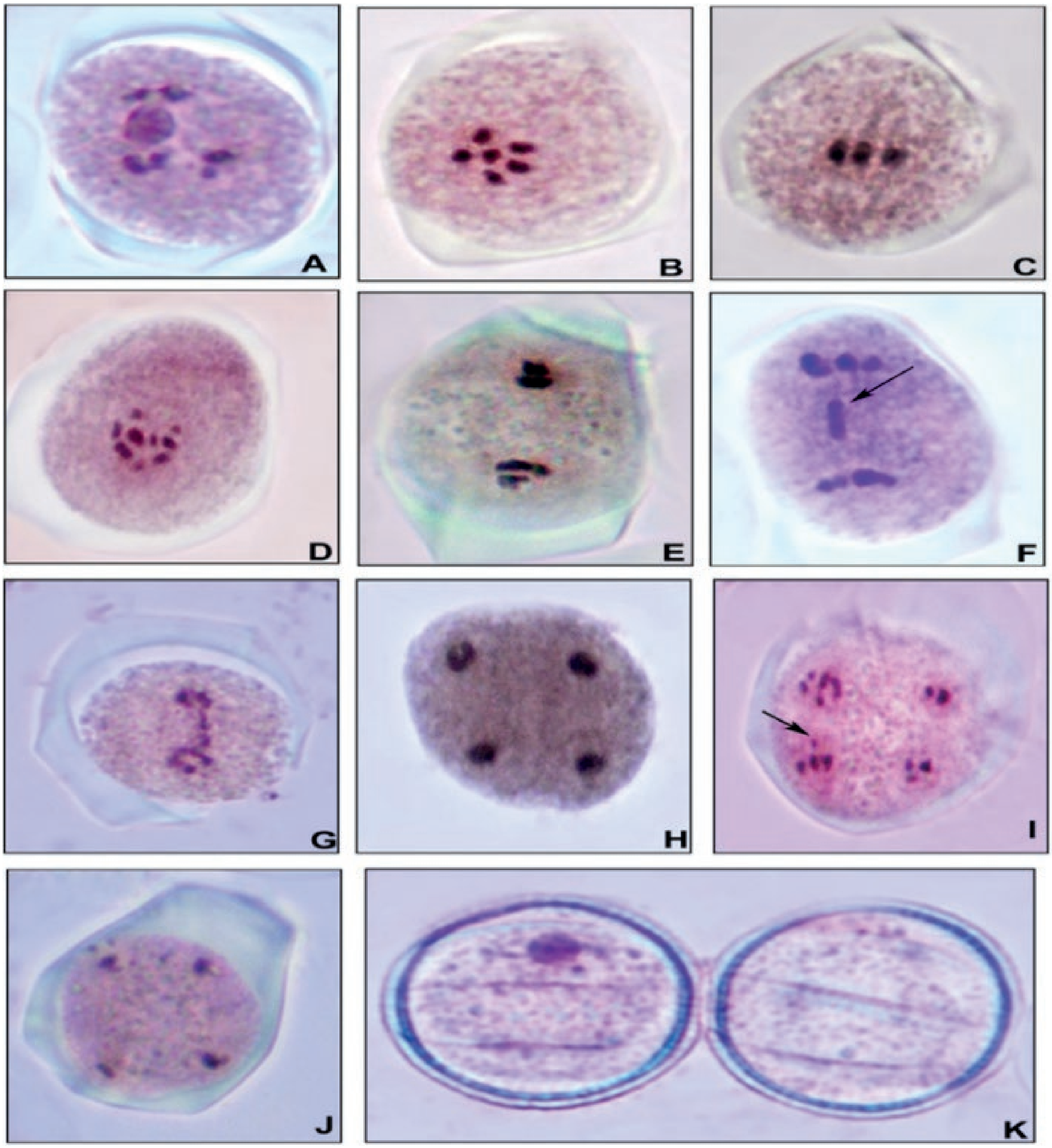

Figure 5. Cytological Meiotic anomalies induced by gamma irradiation: *Salvia hispanica* L. (2n=12) A. Diplotene Stage; B. Normal Metaphase (2n=12); C. Stckiness at metaphase I; D. Scattering at metaphase I; E. Normal Anaphase I; F. laggard at Anaphase I; G. Bridge formation at Anaphase I; H. Normal Anaphase II; I. Laggard at Anaphase II; H. Normal Telophase II; J. Normal Pollen and sterile pollen grains (Scale=10μ).

(60.23%), Linoleic acid methyl ester (19.78%), Palmitic acid (11.96%), vitamin E (5%), and gamma-sitostenone (9.89%) (Table 2). These enhancements in the phytochemical profile were notably higher compared to the control group, representing a novel finding not previously documented in existing literature. In contrast,

Table 2. GC-MS analysis was conducted to compare the concentration percentages and retention times (in minutes) of the treatment and control groups in M2 generation seed of gamma irradiation treatment.

Compound	Metabolites detected	Molecular formula	RT	Control Area %	T1 Area %	T2 Area %	T3 Area %	T4 Area %	T5 Area %
Saturated fatty acids	Myristic acid	C14H28O2	12.53	0.15	0.1	0.19	0.19	0.14	0.13
	Palmitic acid	C16H32O2	14.72	7.52	0.26	8.42	-	5	4.73
	Stearic acid	C18H36O2	16.62	3.67	3.06	3.21	0.33	0.37	1.98
	Beta. Monoglyceride	C19H38O4	19.54	7.27	7.73	6.16	0.14	7.02	6.76
	Stearic acid methyl ester	C19H38O2	16.62	3.67	3.06	3.21	0.18	2.07	0.4
	Lauric acid	C12H24O2	19.91	0.09	0.14	0.28	0.13	0.16	0.1
Unsaturated fatty acid	Linoleic acid, methyl ester	C19H34O2	15.9	1.87	1.18	2.24	0.95	2.28	2.37
	Linolenic acid, methyl ester	C19H32O2	15.97	6	4.52	7.44	7.37	6.9	4.22
	Alpha-Linolenic acid	C18H30O2	16.45	5.46	7.05	17.73	11.6	5.07	0.38
	Linolenic acid, ethyl ester	C20H34O2	21.04	20.2	10.84	13.32	9.45	7.44	4.22
	Alpha-Monostearin	C21H42O4	21.12	2.02	1.7	6.24	1.29	0.17	0.17
	Linolein, 2-mono-	C21H38O4	21.54	0.51	0.08	0.4	0.1	0.37	0.27
	Linolenic acid, methyl ester	C19H32O2	21.61	0.52	0.5	12.59	7.37	4.22	0.3
	Alpha-Linolenic acid	C18H30O2	16.45	5.46	7.05	17.73	11.6	0.38	0.04
Vitamins	Gamma. -Tocopherol	C18H28O3	23.62	1.91	1.81	2.04	1.3	1.81	0.98
	Delta. -Tocopherol	C28H48O2	22.72	0.19	-	6.13	0.17	0.16	0.17
	Vitamin E	C29H50O2	24.34	0.06	0.12	0.36	-	0.16	0.15
Acid ester	Fumaric acid	C25H46O4	17.602	0.82	0.1	0.11	0.37	0.37	0.11
	3-Cyclopentylpropionic acid, 2-dimethylamino ethyl ester	C12H23NO2	19.02	1.56	0.79	0.82	0.71	0.14	1.29
Phytosterol	Stigmasterol	C29H48O	25.91	1.58	0.79	1.04	0.86	1.44	1.59
	Compesterol	C29H48O	25.61	2.09	-	-	-	0.5	0.53
	Gamma-Sitosterol	C29H50O	26.8	8.65	5.26	6.13	9.89	4.53	0.38
	Fucosterol	C30H50O	26.93	0.54	0.34	0.41	-	0.5	0.53
Corticosteroids	11-Dehydrocorticosterone	C21H28O4	21.95	0.3	0.43	0.33	0.44	0.26	0.1

decreases were recorded in the levels of Beta-monoglyceride (15%), 11-dehydrocorticosterone (66.67%), and stearic acid (46%) within the irradiated samples.

It's worth noting that GC-MS analysis was previously conducted by (B. de Falco et al., 2018) under different irradiations of Chia plants; however, their analysis was focused solely on polar and non-polar compounds. The specific response of gamma-treated seeds profiling enhancement in unsaturated fatty acid had not been previously studied. This phenomenon plays a pivotal role in the production of diverse plant varieties.

CONCLUSION

In conclusion, the M_2 generation exhibited more pronounced results across all aspects of the study morphological, cytological, and biochemical when compared to the M_1 generation. The findings suggest that lower doses of gamma irradiation have a stimulating effect on the chia plant's morphological traits and phytochemical properties. The GC-MS analysis of chia seeds showed a notable enhancement in unsaturated fatty acid content at 100 Gy irradiation, which can have positive implications for the plant's medicinal and nutritional properties. However, higher doses were found to be detrimental to these phytochemical properties. This finding is significant as it has the potential to play a pivotal role in enhancing plant productivity and promoting the enlargement of seed size in the treated plants. "The discovery of a notable increase in unsaturated fatty acids, particularly Alpha-linolenic acid, following exposure to 100 Gy of gamma irradiation represents a novel and previously unreported finding. These observations have implications for the potential use of gamma irradiation in crop improvement and seed quality enhancement.

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