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Research Article

Dose optimization, frequency and spectrum of Gamma-ray induced chlorophyll mutations in acid lime (*Citrus aurantifolia*) cv. Agamalai

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Abstract
Citrus, one of the most popular fruit crops grown worldwide, has high nutritional, medicinal and commercial value. This experiment aimed to investigate the effects of gamma radiation with various doses, including 0 Gy, 5Gy, 10 Gy, 15 Gy, 20 Gy, 25Gy, 30 Gy, 35 Gy, 40 Gy and 45 Gy of cobalt 60 sources on acid lime variety Agamalai citrus, (*Citrus aurantifolia*). The study was carried out at the Horticulture College and Research Institute in Periyakulam.The mutagenic efficiency and effectiveness were evaluated using the frequency of chlorophyll mutations and the biological damage in M_1 plants. The stomatal index reduces (19.62 %– 13.99%) with increasing treatment dose, compared to control (20.39%), although guard cell dimensions, such as length (6.09 µm) in 15 Gy and breadth (4.56 µm) in 5 Gy increase with higher dose treatment than untreated (3.71 µm,3.54 µm). Identifying the mutagenic efficiency and effectiveness of the mutagen and maximising the lethal dosage is the first stage in any mutagenesis research. This work will serve as a foundation for subsequent gamma-irradiation studies in acidlime to generate desirable mutants.

Keywords: Agamalai, Cobalt 60, *Citrus aurantifolia,* Effectiveness, Frequency, Mutagen

INTRODUCTION

Acid lime (*Citrus aurantifolia,* Swingle), a member of the Rutaceae family, is often grown in India's tropical and subtropical climate (Rangaraj *et al.,* 2022). Although the exact origin of citrus is unknown, reports indicate it originated in the tropical regions of south and southeast Asia (Kumari *et al.,* 2021). Acidlime, known as nimbu or lebu, is a valuable and versatile fruit, the third most significant fruits followed by mandarin and sweet orange (Prasanna *et al.,* 2023).Globally, the highest acid-lime production is from India (IIFPT, 2021), which has a prominent position in the fruit industry. Fresh fruit is constantly in demand throughout the year, but high prices especially rise in the summer (Sekhar and Kundu, 2021). The *kagzi* lime (*Citrus aurantifolia* Swingle) is an excellent source of minerals such as calcium (90 mg/100 ml), iron (0.3 mg/mg100 ml^{-1}), phosphorus (20 mg/100 ml) and vitamins C (62.90 mg/100 ml), B1, and B2 (Abhilash *et al*., 2018). The area under this crop is rapidly expanding and becoming more important in the citrus industry (Deshmukh *et al*., 2015). Apart from value-added products like pickles, juice, squash, etc., the soap and cosmetic sectors, highly value the lime peel oil and peel powder (Dabhi *et al*., 2023; Akhtar *et al.,* 2024). It plays a key role in the socio-economic condition of small and marginal farmers of India's rainfed and irrigated land areas.

Increasing the yield of acidlime is necessary to meet the growing and expanding population and decline in arable land (Ladaniya *et al*.,2020). Due to the current severe rivalry in producing new fruit (Sathiya *et al*.,2022), growers have been forced to develop new varieties promptly to remain commercially viable (Gidoni and Carmi, 2007).Determining genetic diversity within a plant population is indispensable for successful plant breeding. To achieve this, plant breeders traditionally start with natural genetic variation as a foundation for their hybridization efforts (Swarup *et al*.,2021). This practice encourages the development of a diverse range of plants that are more resilient to various biotic and abiotic stresses (Puripunyavanich *et al*.,2022).

One useful method for improving natural genetic resources is the induction of mutations (Jain, 2002; Holme *et al*.,2019). Mutations are defined as heritable changes that occur in the genetic makeup of an organism, resulting in changes to its characteristics that do not occur by genetic segregation (Devi *et al.,* 2021). Mutations arise from modifications in DNA or from the processes of cell division and replication (Yali and Mitiku, 2022). Ionizing radiation can cause genetic changes and increase the frequency of mutations beyond the rate that occurs naturally (Brunner, 1995), leading to many developments in crop breeding. Electromagnetic radiation, such as gamma rays, X-rays, and UV light and particle radiation, such as beta, alpha particles, fast and thermal neutrons are examples of physical mutagens (Kodym and Afza, 2003). Gamma rays are one of the main radiation sources that cause genetic diversity through mutagenesis (Hong *et al.,* 2022); widely used in fruit crops (Maurya *et al.,* 2022) is prospering in the release of the vast majority of mutant cultivars worldwide (Ghasemi-Soloklui *et al.*,2023).

Physical mutagens have proved effective in generating early flowering, dwarfism and seed lessness in different varieties of fruit crops (Lamo *et al.,* 2017). Mutagenic seed treatment is the most practical and extensively used method for propagating crops from seed because it is simple to handle, store, transport and process in large quantities. Recently, seed radiation has been widely utilised to boost the germination percentage of seeds, enhance the elongation rate in young seedlings, and encourage the growth of different plant parts (Ahmed *et al*., 2018). Radiating the seeds can be utilised in a variety of crops to provide consistent and resilient seedling emergence and establishment. Within reasonable bounds, it is feasible to achieve repeatable results by replicating the mutagenic therapy's pre- and post-treatment conditions (Kodym and Afza, 2003). Morphological characterization contributes to efficiently preserving and managing the currently present genetic diversity. Variable genotypes can then be added to commercial hybrid programmes to create the desired variety with a high yield. (Prasanna *et al*., 2023). In order to maximise the effectiveness of mutation detection, the mutagen dose should be optimised to produce a high rate of mutations to prevent detrimental effects on germination and plant development (Parry *et al.,* 2009; Jency *et al.* 2016; Kumar *et al.,* 2021).

The primary objective of this research was to examine how the acidlime cultivar Agamalai citrus responds to radiation and determine the efficiency and effectiveness of the impact of gamma radiation on physiological status of *A. citrus*.

MATERIALS AND METHODS

This study was conducted to find the impact of gamma irradiation applied as single dosages in acid lime (*Citrus aurantifolia* Swingle) seeds. The investigation was conducted in the Central block, Department of Fruit Science, Horticultural College and Research Institute, Tamil Nadu Agricultural University (TNAU), Periyakulam, from 2023-2024. The variety chosen to undergo induction of radiation treatment was Agamalai citrus, an excellent landrace located in the hilly regions of Agamalai, Periyakulam, in the Theni district of Tamil Nadu. It is widely recognised for its remarkable size, superior juice and unique flavor (Fig. 1). The district has a higher demand for this fruit, driving up costs daily.

The main strategy in mutant breeding is to improve productive and quality plant varieties for well adapting by changing their primary agronomic characteristics. To create variability, well-known physical mutagens gamma rays are used since they induce a higher number of variations due to its deeper penetration into biological substance, higher degree of accuracy and sufficient reproducibility. The mature homogenate fruits from adult trees were harvested in mid-March to obtain seeds for the experiment. The seeds were removed from fruits, cleaned and encased in polythene covers.

Based on the treatment gamma radiation was applied to the acid lime seeds using the ^{60}Co source of different grays from the Indira Gandhi Centre for Atomic Research (IGCAR), Kalpakkam, Tamil Nadu, India, by following proper procedure and protocols. The experiment was determined by complete randomization, a total of 72 seeds were subjected to various gamma irradiation dosages (0, 5, 10, 15, 20, 25, 30, 35, 40 and 45Gy) with three replications. Since the acidlime seeds are recalcitrant, they were sown immediately. The irradiation seeds were placed in polybags containing a 2:1:1 ratio of red soil, FYM, and sand and monitored until the seeds germinated.

The assessment of efficacy and efficiency is of the utmost importance in confirming the use of mutagens in creating beneficial genetic mutations and identifying mutants exhibiting desired traits. Effectiveness shows the mutations caused by a single mutagen dose. Mutagenic efficiency describes the frequency of mutations responding to biological damages, such as injury to seedlings and anomalies in meiosis generated in the M_1 generation. The chlorophyll mutants are subsequently characterized, and the mutagenic efficacy and efficiency of gamma rays were ascertained using the following formula.

Mutation frequency (M1 plant basis) = Number of chlorophyll mutants/ Total number of plants X 100 Eq. 1

Mutation effectiveness = Mutation frequency / Dose of mutagen (Gy) x 100 Eq. 2

Mutation efficiency= Mutation frequency / Percent lethality (L) x 100 Eq. 3

(or)

= Percent injury (I) or Percent sterility (S)

Physiological analysis

The stomatal characteristics of the newly matured leaves were painted with nail polish on their lower surface and allowed to dry completely before taking accurate impressions. The leaf polish patch was then delicately peeled off to reveal the leaf imprint, which was then examined under 10X and 40 X Florescent microscopes and the stomatal index was determined using the formula.

Stomatal index = Stomatal index (%) = (S/S+E) x 100 Eq. 4

S - Number of stomata

E - Number of epidermal cells

Types of chlorophyll mutants

Mutant	Description
Albina	White-colored foliage
Xantha	Leaves with a yellow colour.
Chlorina	Leaves with a light green colour
Alboviridis	Initially white, then transforms into a normal plant

In the M_1 generation, biological harm like injury, mortality and the emergence of chlorophyll mutants were noted. Using probit analysis, gamma irradiation's lethal dose (LD_{50}) values were determined based on the germination percentage.

RESULTS AND DISCUSSION

The present study observed that gamma radiation at varying doses impacted the plant development parameters during the vegetative phase. The seed germination test after gamma irradiation revealed that the control seeds had the highest germination percentage (93.05%) followed by (84.72%) compared to the irradiated seeds in 5 Gy and the germination percentage decreased to (6.94%) when gamma ray exposure increased for 45 Gy (Table 1). This is because gamma radiation negatively damages the germination ability of the seeds in a dose-dependent manner (Ma *et al*.,2023). The harmful effects of higher doses of gamma rays may be the reason for the reduction in sprouting, whereas lower levels of the same radiation accelerated metabolic activity. An increase in dosage results in a decreased survival rate (40%) at 45 Gy than control (93.05%) similar findings reported in grapes (Coban *et al.,* 2002). This might be the reason that gamma radiation at lower dosages promotes cell division, growth and development. Similar results were elicited by Agisimanto *et al.* (2016) in *Citrus reticulata* cv. Limau Madu following exposure to a low dose of 10 Gy gamma radiation. The reductions in growth parameters above 15 Gy are also due to disrupting regular mitosis and causing numerous aberrations, slowing down the process of assimilation and changing the nutrients that plants receive (Ramesh *et al.,*2012; Ghasemi-Soloklui *et al*.,2023).

Optimizing the lethal dose, often denoted by LD_{50}, is critical in mutagenesis research as it enables the identification of the irradiation dosage that results in a 50% mortality rate within a specified time frame and in present research, the LD_{50} for Agamalai citrus was 26.51. While distinct development features were produced in seedlings by lower doses of gamma rays, this is typi-

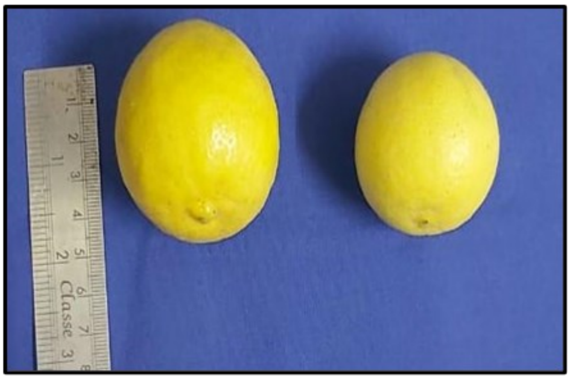

Fig. 1. *Size comparision of Agamalai with PKM1*

Table 1. Probit analysis of acid lime variety Agamalai citrus

Dose (Gy)	Log_{10} of doses	Germination percentage	Percent reduction over control	Observed mortality percentage	Corrected mortality percentage	Empirical probit units	LD_{50} value
0(Control)		93.05		6.94	0.00		
5	0.70	84.72	8.33	15.28	9.0	3.66	
10	1.00	79.16	13.89	20.83	14.9	3.96	
15	1.18	70.83	22.22	29.17	23.9	4.29	
20	1.30	65.27	27.78	34.72	29.9	4.47	
25	1.40	61.11	31.94	38.89	34.3	4.60	
30	1.48	41.66	51.39	58.33	55.2	5.13	26.51
35	1.54	18.05	75.00	81.94	80.6	5.86	
40	1.60	11.11	81.94	88.89	88.1	6.18	
45	1.65	6.94	86.11	93.06	92.5	6.44	

cally due to the reduced mutation frequencies (Mba *et al*., 2010). All the biological parameters, including mortality, sterility and seedling damage, occur when gamma irradiation increases to 35 Gy. Gamma irradiation's mutagenic efficiency and effectiveness were maximum at LD_{50} and then declined as dosages increased above 30 Gy (Table 2). Lower doses of gamma radiation (5Gy -15) stimulated some growth features, but higher levels (20 Gy - 45 Gy) had a detrimental effect.

In mutation breeding, chlorophyll mutants are undesirable characters but are crucial indicators of mutagenesis efficiency (Table 3). Furthermore, within the irradiated population, accessible mutations can be identified through the anomalies in chlorophyll deficiency caused by irradiation. The chlorophyll mutants of xantha, chlorina, albino and Albinoviridis are among the chlorophyll deficient mutation variations discovered during this investigation; the most prevalent type was Alboviridis (Fig. 4). This is due to the photosynthetic complex that is altered by ionising radiation, lowering the efficiency of the photosystem (Pramanik *et al.,* 2023). The chlorophyll mutants may arise from various causes, including deficits in carotenoids, incorrect chlorophyll synthesis and chlorophyll degradation. These findings matched those of (Ravi *et al.,* 2022) in papaya. The stable chlorophyll mutants with favourable agronomic traits can be employed promptly as breeding material to

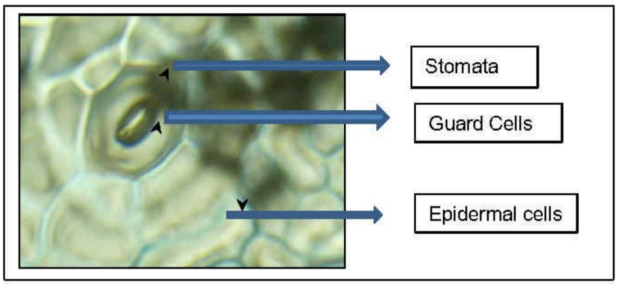

Fig. 2. *Slender paradermal segment of an adult stoma and subsidiary cell*

create novel genotypes. Regarding the other chlorophyll mutants, the frequency increased to 30 Gy but drastically decreased after 35 Gy of gamma-ray exposure. This could be because higher dosages of physical mutagen are associated with mortality.

Stomata have evolved to prevent dehydration and physiological harm by balancing maximum carbon uptake and minimal water loss (Rogiers *et al.,* 2012). Plant survival and function depend on stomata, tiny pores or valves that regulate the entry of carbon dioxide taken up during photosynthesis and optimise water efficiency (Sanchez *et al.,* 2013). The microscopic stomata structures comprised two specialist kidney-shaped guard cells surrounding a central pore (Fig. 2). The number of stomata varied among plant species that exhibit a broad range of shapes, sizes and numbers

In the present study on Acidlime variety Agamalai, the leaves had maximum stomata on their lower epidermis (abaxial) (563 µm –796µm) compared to the upper (adaxial) side (12 µm - 57 µm) and distributed unevenly (Fig 3). The function of the stomata is to control gases that enter and exit the leaves, thereby regulating the temperature inside the leaf and controlling the amount

Table 2. Mutagenic *efficiency* and *effectiveness* of Agamalai citrus treated with different dose of gamma rays

SP	SRP	ME	MEF
93.05	--	--	--
91.80	1.25	525.38	1.31
87.72	5.33	131.50	0.70
82.35	9.45	82.54	0.52
78.72	9.00	70.92	0.31
77.27	15.78	57.61	0.36
73.33	19.72	33.83	0.22
69.23	23.82	32.28	0.25
50.00	43.05	0.00	0.00
40.00	53.05	0.00	0.00

SP-Survival percentage, SRP- Survival reduction percentage, ME-Mutagenic efficiency, MEF-Mutagenic effectiveness

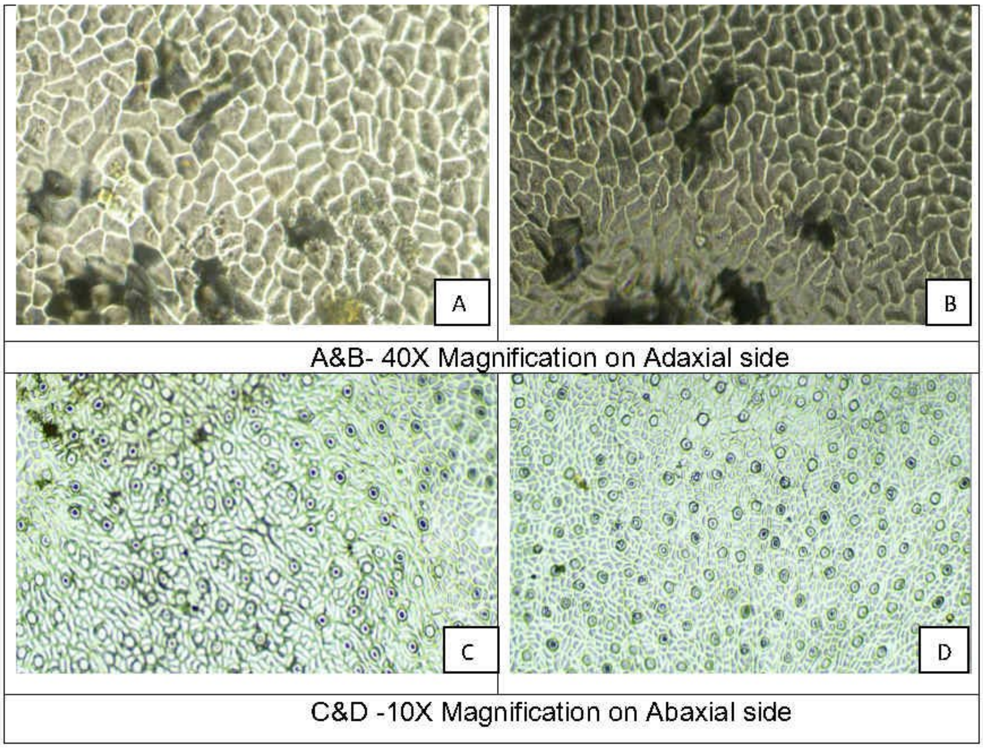

Fig. 3. *Microscope view of Stomata A & C in Control plants and B & D in mutated plants*

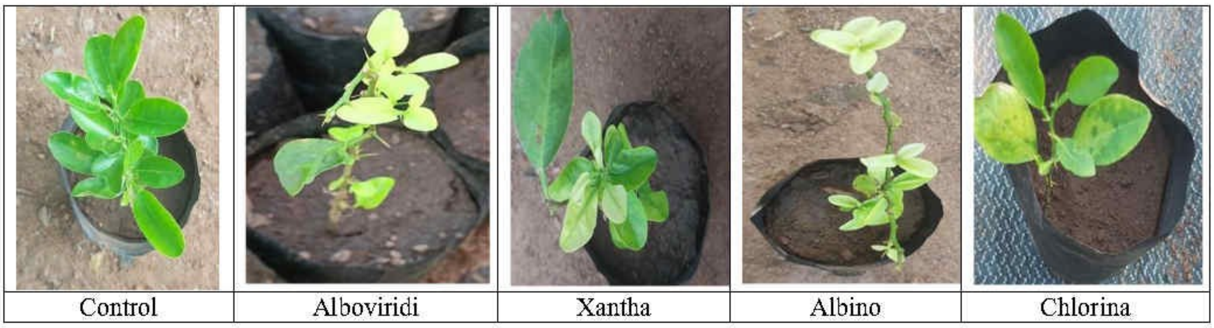

Fig. 4. *Chlorophyll deficient mutants*

Table 3. Mutagenic frequency of Agamalai citrus at different dosages of gamma rays

Dose (Gy)	Total number of plants	Number of chlorophyll mutants					MF
		Xantha viridis	Albino Viridis	Albina	Chlorina	Total	
0 (Control)	67						
5	61	-	4	-	-	4	6.55
10	57	1	6	-	-	4	7.01
15	51	-	4	1	-	5	7.80
20	47	-	3	-	-	3	6.38
25	44	-	4	-	-	4	9.09
30	30	-	2	-	-	2	6.67
35	13	-	-	-	1	1	7.69
40	8	-	-	-	-	0	0.00
45	5	-	-	-	-	0	00.0

MF- Mutation frequency

of transpiration. In Agamalai citrus, stomata were typically seen to be paracytic (Table 4). The paracytic stomata is a mature stomata with a distinctive pair of lateral subsidiary cells oriented parallel to the guard cells (Cullen and Rudall, 2016) and very different from neighbouring cells in terms of shape. There was no discernible relationship between the stomatal density and the length or width of the stomata. Gamma irradiation had a greater ability to alter the stomatal features. The stomatal index reduced from 19.62 % in 5 Gy to

Table 4. Difference in stomata number with respective to gamma rays dosages

Dose (Gy)	Stomatal complex type	No of stomata/mm^2		Stomata length	Stomatal breadth	Stomatal index (%)
		Adaxial	Abaxial			
0(Control)	Paracytic	57	796	3.71	3.54	20.39
5	Paracytic	21	753	4.43	4.56	19.62
10	Paracytic	16	683	4.81	1.52	18.24
15	Paracytic	13	579	6.09	3.32	15.63
20	Paracytic	18	596	5.14	4.49	14.18
25	Paracytic	12	563	5.17	4.43	13.99

13.99% with an increasing treatment dose of 45 Gy, compared to control (20.39%), although guard cell dimensions, such as length (6.09 µm) were highest in 15 Gy and breadth (4.56 µm) in 5 Gy increase with irradiated treatment than untreated (3.71 µm,3.54 µm). The changes in stomata index is due to external climatic factors, light, water, humidity and temperature and internal factors like leaf thickness, stomatal density, presence of cuticles, as well as the shape and positioning of stomata on the leaf surface (Nyainleta *et al*.,2022). As dosage levels increased, there was an overall decrease in stomatal density and an increase in stomata length-width. This is because gamma irradiation causes genetic changes that decrease the amount of free auxin available for tissue development and could have a suppressive influence on stomatal density (Maana and Brar, 2021). A reduction in stomatal density concerning leaf area was the indicator of dwarfism. One important factor influencing stress tolerance that may help plants adapt to salinity more successfully is decreased stomatal density and the resulting constitutively reduced transpiration flux (Orsini *et al.,* 2012).

Conclusion

Increasing gamma ray dosage results in a linear decrease in growth metrics such as germination, survival and difference in difference in stomata. The highest Stomatal index (20.39%) was in control, followed by **(**19.62%) in 5 gy and the lowest at 25 gy with (13.99%). Similarly, stomata length and breadth were higher in irradiated plants than in the control. The formation of chlorophyll mutations was the most noticeable and easily identifiable. They are widely employed to assess crop plant sensitivity to mutagens and determine their effectiveness and efficiency. The LD_{50} value was determined to be 26.51 and the research results confirmed that gamma irradiation produced considerable changes in Agamalai citrus. Therefore, the completed study will provide a basis for ongoing observation to monitor and select the most promising mutants for subsequent M_2 generations. This will offer valuable insights and resources for the plant breeders in the future.

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Conflict of interest

The authors declare that they have no conflict of interest.

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