MIJST
MIST International Journal of Science and Technology

Visual Assessment and Genetic Distance Analysis of Gamma-Irradiated Lemon Leaf Mutants Developed Using the Leaf-Cut Method

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ARTICLE INFO

Article History:

Received: 26th May 2025
Revised: 07th September 2025
Accepted: 28th September 2025
Published: 30th December 2025

Keywords:

Gamma radiation
Lemon leaf mutant
Leaf-cut propagation
Genetic distance
Visual inspection

ABSTRACT

Ionizing radiation, particularly gamma rays, is widely used to induce genetic mutations for improving morphological traits and enhancing genetic diversity in plants. In citrus plants, such mutations can be achieved by irradiating leaves and propagating the resulting mutants through vegetative methods. This study was conducted at the BINA research farm with the objective of developing Citrus limon mutants using gamma radiation and analyzing their morphological characteristics through visual inspection, alongside genetic diversity assessment using hierarchical clustering. A total of 400 fresh leaves were collected from the mother plant (BINA Lebu-1) and exposed to different doses of gamma radiation (0, 60, 80, and 100 Gy), divided into four treatment batches. The irradiated samples were planted in unit plots following a Randomized Complete Block Design (RCBD). Root and shoot development were monitored visually after 3 to 4 months. Maximum root length was observed at 100 Gy, the highest root quantity at 60 Gy, and the highest success rate at 80 Gy. After 7–8 months, mature plants were further assessed, and leaves were collected for genomic analysis. Genetic relationships among the different treatment groups were evaluated using a Dendrogram generated through simple hierarchical clustering. The results indicated clear genetic dissimilarities between irradiated (60, 80, and 100 Gy) and non-irradiated (0 Gy) plants, with the 100 Gy group showing the greatest genetic distance from the others. Due to time constraints, fruit production could not be observed. Nonetheless, the study demonstrates the effectiveness of combining visual inspection and genetic distance analysis in evaluating gamma-induced citrus mutants developed via the leaf-cut method.

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1. INTRODUCTION

Lemon (Citrus limon) is the best source of vitamin C and contains other essential nutrients that are good for human health. Lemon aids in improving human health with countless benefits (Barkley et al., 2006). Citrus fruits like lemon play a vital role in agriculture as their demand is increasing with the growth of the population (Hodgson, 1967). However, conventional breeding techniques of citrus face multiple challenges, such as limited efficiency, climate conditions, etc. Mutation induction techniques such as radiation effectively overcome these challenges, typically arising from extremely low-frequency spontaneous mutations, by increasing variability in plant characteristics (Belele et al., 2001; Çimen et al., 2020). Gamma radiation induces DNA damage and structural and physiological alterations; this can result in new variations like agronomic or molecular differences (Sparrow et al., 1956; Whittwer, 1971). The leaf-cut technique is an asexual propagation method that allows roots and shoots to form at the base of leaves. This method maintains consistency in yield and formation of genetically identical plants (Deependra et al., 2018). Visual inspection is used to observe and record morphological variations from irradiated gamma lemon leaf mutants (Waqar et al., 1992). Cluster analysis is employed to group genetically similar and dissimilar plants into clusters (Gulsen et al., 2001; Sidler et al., 2015). This technique can help us to discover genetic distances or changes in lemon plants at different gamma levels. Genetic distance offers more opportunities for the conservation and evaluation of genetic resources (Taheri et al., 2014). This research aims to screen for better lemon cultivars by examining the impact of gamma radiation on the leaves of lemons formed through leaf-

cutting. This study offers farmers a shorter path to obtain improved lemon plants than traditional breeding (Kumar et al., 2013). This study also has relevance for agricultural biotechnology because it examines a low-cost and scalable method for genetically improved lemon propagation.

2. MATERIALS AND METHODS

An experiment was conducted at Horticulture research farm of Bangladesh Institute of Nuclear Agriculture (BINA), Mymensingh, from July 2024 to April 2025, to induce mutation by gamma radiation in *Citrus Limon* using leaf cut propagation technique.

2.1 Sample Collection and Separation

Lemon leaves were collected from non-irradiated mother plant (BINA Lebu-1). Four hundred fresh leaves were gathered and separated into 4 batches; each batch consisted of 100 leaves. Then, the samples were labeled according to desired doses (60, 80 and 100 Gy) before exposing them to radiation, shown in Figure 1. One of the batches was labeled as a control batch (0 Gy), which would be unexposed to radiation.

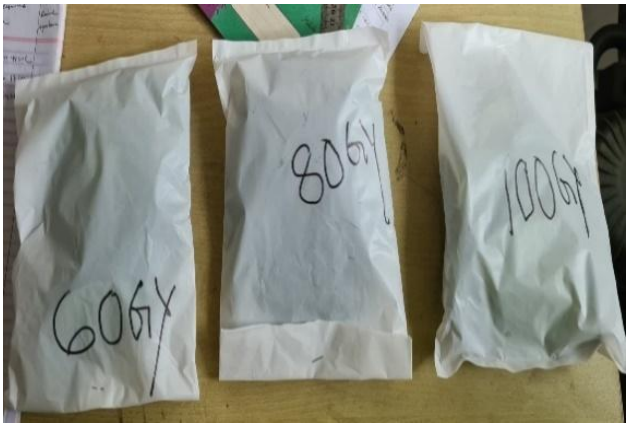

Figure 1: Sample (BINA Lebu-1 leaves) separation according to desired radiation doses

2.2 Exposure data calculation and Irradiation

Required exposure time was calculated before irradiation of the samples. For radiation exposure on leaves, a shielded gamma irradiation facility, known as a gamma chamber shown in Figure 2, was used. Co-60 used as a radiation source in the gamma chamber. After calculating the required parameters, a single batch of samples each time, totaling 3 batches were placed and irradiated in the chamber.

Figure 2: Irradiator Co-60 Gamma Chamber (Model-GC-5000)

2.3 Planting and Monitoring

After irradiating the leaves, cutting aid (containing Indole Butyric Acid, Poly Hydroxyl Butyrate and boric acid) was applied as a root promoter to the leaves and planted in the prepared soil at a shade house. The leaves were planted in the RCBD (Randomized Complete Block Design) with 3 replications. After planting all the leaves, a suitable environment (Temperature: 25-35°C and relative humidity: 80-100%) should be provided in the shade house to promote the formation of callus, roots, and shoots.

2.4 Morphological Assessment

A few days after planting, the initiation of roots was observed. The length of the roots was measured for three levels of gamma doses at 60, 70, and 90 days after planting. After the formation of shoots, shoot lengths were also measured at 90, 120, and 150 days after planting. Other notable changes were also recorded. Figure 3 shows the formation of roots from the irradiated leaves for different gamma levels.

Figure 3: Root formation from irradiated leaves (60,80 and 100 Gy from left to right)

2.5 Molecular Data Acquisition

After 7 to 8 months of planting, grown plants were observed. Then, leaves were detached from them to analyze genomic data. To evaluate the plant genomic data, DNA Extraction & Gel Electrophoresis Test were conducted.

2.5.1 DNA Extraction

To extract DNA from plant tissues, the leaves detached from plants grown under radiation treatments (0, 60, 80, 100 Gy) were grinded and mixed with liquid nitrogen and preheated extraction buffer and poured into 4 different micro centrifuge tubes. Then, they were centrifuged at 13,000 rpm for 12 minutes, for 3 times and after discarding the supernatant and drying, concentrated DNA pellet samples for each tube was obtained.

2.5.2 Gel Electrophoresis Testing

Before gel electrophoresis test, DNA pellets were amplified by RAPD-PCR (Random Amplified Polymorphic DNA- Polymerase Chain Reaction) process. The samples were prepared with the mixture of concentrated DNA pellets, RAPD primer (Cp-02), PCR master mix (polymerase, buffer) were placed in PCR Thermal Cycler machine and rotated for 45 cycles to automate DNA amplification. After amplification, Gel electrophoresis or RAPD PCR gel run test, shown in Figure 3, was conducted to separate DNA fragments by applying an electric field to an agarose gel matrix. After running the gel along with a 1 kilo base (kb) DNA ladder as a reference for size estimation of DNA fragments, it was stained with a dye which was Ethidium Bromide (EtBr) and visualized under UV light. The DNA fragments appeared as bands and to observe the band pattern, a gel documentation system or a camera was used to capture an image of the gel, including the ladder.

2.6 Cluster Analysis

Analysis of the gel image showed different bands for each sample, representing unique DNA fragments. These bands were noted as "1" (band present) or "0" (band absent) for statistical analysis. Then, a similarity matrix is created, using Jaccard's coefficients to compare band patterns. Based on Jaccard distance between each sample, a simple hierarchical clustering was constructed. The result is visualized as a Dendrogram, where branch lengths represent genetic distance.

3. RESULTS AND DISCUSSIONS

3.1 Morphological Variation Inspection

For the different levels of gamma irradiation, significant morphological variations were found per leaf cutting. The average time of callus formation, time of root initiation, and time of shoot initiation was recorded for all gamma levels, shown in Figure 4.

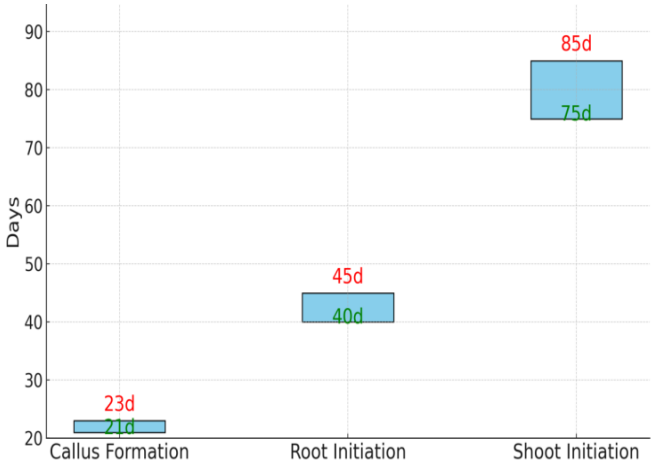

Figure 4: Timeline for different stages of plant growth

Figure 4 shows that the timeline for callus formation was 21 to 23 days after planting. So, the average time of callus formation was 22 days. The timeline for root initiation was 40 to 45 days after planting. So, the average time of root initiation was 42 days. And the timeline for shoot initiation was 75 to 85 days after planting. Therefore, the average time of shoot initiation was recorded to be 80 days after planting of leaves. Then, the number and length of roots, length of shoots, plant survivability and success rate were recorded, shown in Table 1.

Table 1: Combined morphological variation on different gamma levels

Table 1 shows the rate of success and survivability is higher for low doses, and the success rate decreases as the dose level increases. For the 100 Gy dose, the survivability rate is minimum. And the maximum morphological changes are observed for 100 Gy than for other doses. While the maximum number of roots and minimum length of roots and shoots is found for 60 Gy. And average morphological changes are found for the 80 Gy dose.

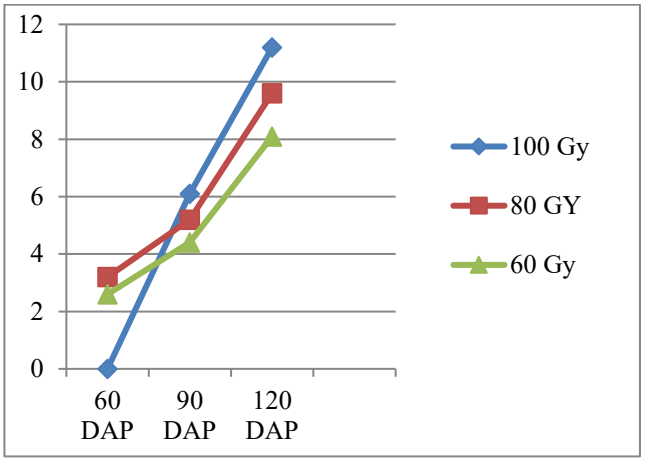

Figure 5: Length of roots on different gamma radiation levels

Though the highest length of root (11.2 cm) and shoot (25 cm) was found for the 100 Gy dose, (Figure 5 and Figure 6), the percent survivability and percent success are minimum for 100 Gy. Maximum survivability (43.76) and success rate (39.44) were found for the 60 Gy dose (Figure 7).

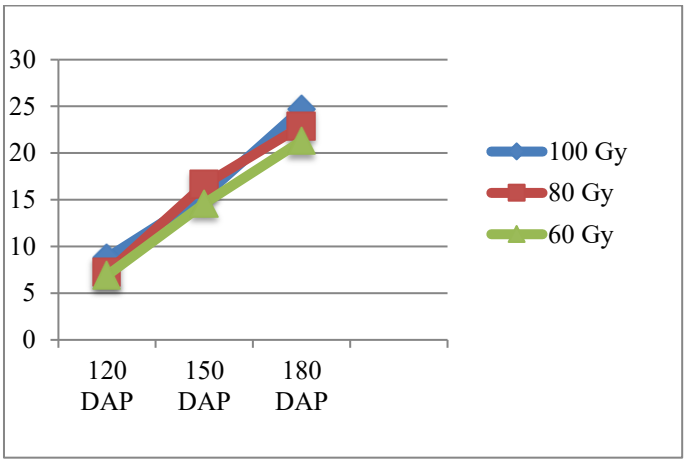

Figure 6: Length of shoots on different gamma radiation levels

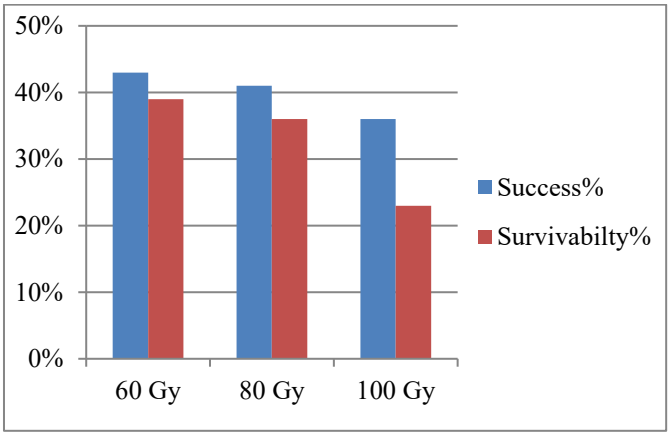

Figure 7: Percent success and survivability of propagated lemon leaves on different irradiation level

3.2 Genetic distance Measurement

From the gel image, band patterns indicated the degree of band presence and absence for samples of different radiation doses. Table 2 shows the values of band presence and absence obtained for different samples in a range of 2000-100 band pattern(bp).

Gamma Dose	Number of Roots			Length of Roots (cm)			Length of Shoots (cm)			Survivability (%)	Success (%)
	60 DAP	90 DAP	120 DAP	60 DAP	90 DAP	120 DAP	120 DAP	150 DAP	180 DAP		
100 Gy	3-5	4-6	6-8	0	6.1	11.2	8.8	15.4	24.7	38.21	25.78
80 Gy	1-3	3-4	4-7	3.2	5.2	9.6	7.2	16.6	22.9	42.33	34.96
60 Gy	2-4	2-6	5-10	2.6	4.4	8.1	6.9	14.6	21.3	43.76	39.44

Table 2: Binary Matrix (Band Presence/Absence)

Band (bp)	100 Gy	80 Gy	60 Gy	0 Gy (control)
2000	1	0	0	0
1000	1	1	1	1
750	1	1	1	1
500	1	1	1	0
250	0	0	0	0
100	0	0	0	0

Here, 0 = Band absent; 1 = Band present

To determine genetic distances, Jaccard Similarity Coefficients are to be calculated. The Jaccard index measures similarity between samples. Here, it was used to measure genetic similarity between different samples (0,60,80,100 Gy).

The formula for measuring the Jaccard Index,

$$\mathbf{JI} = \frac{\textbf{Shared bands}}{\textbf{Total bands present in the sample}}$$

The Jaccard index is a coefficient, so it is a dimensionless quantity.

Then, the next thing to be measured was the Jaccard distance. It indicated the degree of genetic dissimilarity between different samples.

The formula for measuring Jaccard distance,

JD = 1- Jaccard index (JI).

Jaccard distance is also a dimensionless quantity.

Table 3 shows the calculated values of the Jaccard index and the Jaccard distance.

Table 3: Calculation of Jaccard index and Jaccard distance

Sample Pair	Shared Bands	Total Unique Bands	Jaccard Index	Jaccard Distance
100 Gy vs 80 Gy	3	4	0.75	0.25
100 Gy vs 60 Gy	3	4	0.75	0.25
100 Gy vs 0 Gy	2	4	0.50	0.50
80 Gy vs 60 Gy	3	3	1.00	0.00
80 Gy vs 0 Gy	2	3	0.67	0.33
60 Gy vs 0 Gy	2	3	0.67	0.33

The higher values of Jaccard distance indicate less similarity. Genetic distances are calculated by averaging the Jaccard distances between the most similar samples and clustering them together. As the Jaccard distance between 80 and 60 Gy is lower than that of others, both of them are the most similar samples. So, these two were clustered first. Similarly, the next closest sample was clustered with the previous one and the genetic distance between them was calculated by averaging their individual distances. For a cluster of (100/80) - 60 Gy, the genetic distance will be
(0+0.25+0.25)/2 = 0.25. Similarly, all the given samples were clustered.

Table 4 shows the clustering of samples with their genetic distances, given below:

Table 4: Clustering of samples with their genetic distances

Cluster of samples	Genetic Distance
80 Gy - 60 Gy	0.00 (~0.01 for visual adjustment)
(100/80) - 60 Gy	0.25
(100/80/60) - 0 Gy	0.39

As the genetic distance between 80 and 60 Gy is almost 0, 0.01 was taken 0.01 instead of 0 for visual adjustment in the illustration of the Dendrogram.
Now, the Dendrogram shows the relationship between Genetic Distances in different samples.

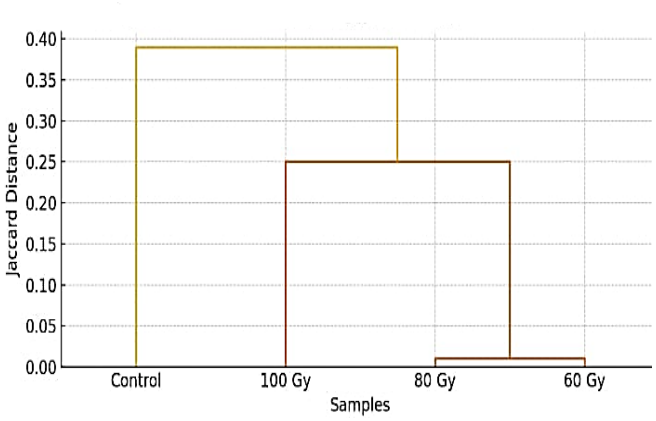

Figure 8: A simple Dendrogram Construction

In the above figure, X axis shows the samples to be compared with each other and Y axis shows the height at which clusters merge. Hence, the Dendrogram indicates that control (0 Gy) or non-irradiated samples have large genetic distance with others. It means, gamma radiation successfully produced mutations in samples of doses 100, 80 and 60 Gy. However, if the samples of 100, 80 and 60 Gy doses are compared, 60 and 80 Gy samples are mostly similar and 100 Gy have larger genetic distance with 80 and 60 Gy. Therefore, these results suggest that increasing radiation doses amplifies its genetic distance.

4. CONCLUSIONS

This study provides a comprehensive analysis of induced mutations in *Citrus limon* through the combined application of the leaf-cut technique and gamma irradiation. The dual approach—visual inspection of morphological traits and genetic distance analysis—proved effective in identifying and distinguishing mutant lines. Among the gamma radiation doses tested, 60 Gy appeared to be the threshold dose for mutation induction, 80 Gy was found to be the most optimal for successful plant regeneration, and 100 Gy induced the highest degree of genetic variation. These findings demonstrate the potential of gamma irradiation, particularly at controlled doses, for generating genetic diversity in citrus plants for future breeding programs.

ACKNOWLEDGMENTS

The authors would like to thank the Military Institute of Science and Technology (MIST) and the Bangladesh Institute of Nuclear Agriculture (BINA) for their guidance, support, and provision of laboratory facilities.

DATA AVAILABILITY STATEMENT

Datasets generated during the current study are available from the corresponding author upon reasonable request.

FUNDING DECLARATION

This research was self-funded.

ETHICS APPROVAL

This study is an engineering experimental investigation. The MIJST Research Ethics Committee has confirmed that formal ethical approval was not required.

ETHICS, CONSENT TO PARTICIPATE, AND CONSENT TO PUBLISH

Not applicable.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS

Author 1: Jawata Afnan - Conceptualization, methodology, experimental investigation, data analysis, visualization, and manuscript writing (original draft).

Author 2: Moriom Azad - Experimental investigation, data collection, data curation, and assistance in analysis.

Author 3: Mokhlesur Rahman - Supervision, resources, review, and editing of the manuscript.

ARTIFICIAL INTELLIGENCE ASSISTANCE STATEMENT

Portions of this manuscript were assisted by an artificial intelligence language model (ChatGPT, OpenAI). The tool was used solely for language editing, text refinement, and clarity improvement. All content, data interpretation, analysis, conclusions, and final decisions were generated, verified, and approved by the authors. The authors take full responsibility for the accuracy and integrity of the manuscript.

CONFLICT OF INTEREST DECLARATION

The authors declare that they have no conflicts of interest.

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