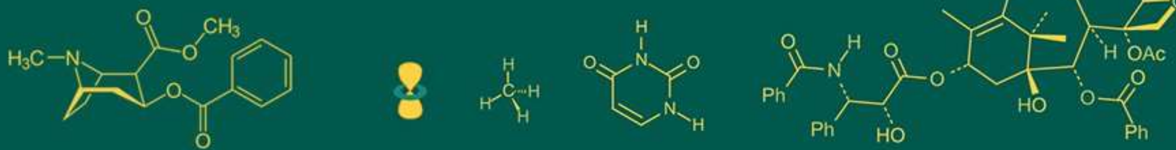

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Improvement of red banana (*Musa acuminata*) through gamma ray: Determination of gamma ray dosage sensitivity and genetic exploration of dwarf traits through molecular marker

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Abstract
Red banana is one of the favorite banana cultivar known for its taste and nutrition. Induced mutation technique is particularly important for bananas as there is limited sexual reproduction and parthenocarpic fruits. In this study, *in vitro* shoots of red banana were treated with gamma radiation (^{60}Cobalt). The objective was to determine the gamma ray dosage sensitivity (lethal dose-LD_{50}) and identification of dwarf plants through molecular marker in red banana variety. The axenic cultures of passage 3-4 were irradiated with doses of 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 Gray (Gy). *In vitro* cultures were evaluated for percent survival, multiplication rate, length of shoots, number of leaves and length of leaves. Based on the values, the lethal-dose 50% (LD_{50}) was determined as 20 Gy. Increasing levels of gamma irradiation doses more than 30 Gy, causes shoots turned to brown and simultaneously dead. Medium supplemented with 6 mg/l 6-benzylaminopurine (BAP) + 20 mg Casein hydrolysate + 30 mg/l Adenine sulphate showed maximum rate of *in vitro* multiplication rate (1:1.8) in irradiated cultures. Total 3,200 irradiated plants grown through micropropagation and their molecular analysis carried out through Sequence Characterized Amplified Regions (SCAR) markers. Molecular analysis revealed the presence of dwarf gene in 14, 18 and 52 sample plants as well as morphological study shows plant height 287cm , 296cm and 282cm, respectively.

Keywords: Red banana, gamma irradiation, cobalt-60, *in vitro*, ld_{50}, scar markers

Introduction

Banana is a globally 4th most important food commodity with a great socio-economic importance in tropical and subtropical regions of India [1, 2]. India is top country of banana production in the world. As of 2020, bananas production in India was 31.5 MT which accounts for 26.47% of the total world's bananas production. The world's total bananas production was estimated at 119 million tonnes in 2020. In India, banana occupies approx. 20% of area from the total area under crop [3]. The estimated area available for the production of banana across India was approx.923 thousand hectares with a production of 33.37 MT (fiscal year 2021) [4].

Red banana (*Musa acuminata*) a triploid-AAA Group variety has great demand because of its taste and nutrition [4, 5]. Its fruit provides number of health benefits such as strengthens immune system, relieves heart health, improves eye and skin health and alleviates digestive problems. On an average one banana provides 15% of the vitamin C, 20% of the daily recommended amount of vitamin D, 11% of the potassium and 16% of the dietary fibre needed each day for good health [6, 7, 8]. They also rich in beta-carotene, antioxidants and vitamin-B6 than any other fruit [9].

Red banana is clonally propagated and it is difficult to breed this triploid variety using hybridization methods which hampers the improvement for this variety [10]. Red banana plants are tall and susceptible to lodging and hence there is a need to develop dwarf statured plants. Dwarfism in red banana will protect the plants from gusty winds. Induced mutation has a high potential to bring genetic improvement in banana plants. During gamma irradiation the mutation rate is based on the time the cells/tissues are subjected to the gamma

as well as the dosage. In this study, ^{60}Co gamma irradiation used to induce mutations in red banana. Combinational use of *in vitro* culture and induced mutation is one of the suitable approaches to improvement of this important red banana [11, 12].

Plant tissue culture provides direct shoot differentiation and number of *in vitro* plant cultures can be subjected to mutagenic treatments [13, 14]. The success of any *in vitro* mutagenesis programme depends on the establishment of reproducible *in vitro* plant regeneration procedures, optimization of mutagenic treatments and efficient screening of the mutagenized populations for desired characters. Gamma radiation technique with *in vitro* propagation, provide healthy starting plant material [15, 16, 17]. has been found to be effective in terms of acquiring variation; rapid proliferation of mutagenized plant population [18, 19, 20]. However, during such attempts optimizing the level of dose becomes crucial especially for *in vitro* culture derived plants as they are highly sensitive. The radio-sensitivity of *in vitro* plantlets is usually estimated through the physiological response of the irradiated material [21, 22].

DNA molecular markers usedto enabling the identification and selection of genes linked to specific traits. This research aimed to perform selection of dwarf growth traits from population of M_1V_5 red banana obtained by gamma rays irradiation.

Materials and Methods

Source of explants

The study were conducted from 12thNov, 2020 to 31st March, 2023 in Plant Biotechnology Centre, College of Agriculture, Dr. Balasaheb Sawant Konkan Krishi Vidyapeeth, Dapoli-415712, Dist-Ratnagiri, Maharashtra State, India. Healthy plant material obtained from field grown suckers of research station Rukhi (Central Research Station, Wakawali, Dr. BSKKV, Dapoli, Dist-Ratnagiri). Quality suckers about 40cm-50cm height were collected from the selected mother plants in November, 2020. After collection of sword suckers these samples were brought to the laboratory and sizing done with clean knife.

Surface sterilization of explants

Shoot meristems were prepared following the protocol described by Sawardekar and Sherkar (2021) [23]. After removing the outer sheaths of suckers, they resized to 8cm (height) to 6 cm (width) of pieces. These suckers were kept under running tap water (for 30 minutes.) followed by the pre-treatment of tween-20 (1%) for 10 min and savlon (1%) for 10 min. These suckers were surface sterilized using various sterilants in laminar air flow. Explants were treated with different sterilants for different durations as mentioned in Table-1. After Surface sterilization these explants were rinsed 3-4 times with sterile distilled water. The shoot tip with a meristem was cultured on nutrient medium.

Media preparation

During *in vitro* studies of cultures, Murashige and Skoog (MS-1962) [24]. medium used. After addition of Plant growth regulators pH of the medium was adjusted to 5.85 with 1N NaOH and/or 1N HCl. The medium was then boiled, gelling agent-agar was added for solidification of the medium and dispensed in culture glass jar bottles. Medium autoclaved at pressure 15 lbs /inch^{2}at temperature of 121℃ for 20 minutes for sterilization.

Establishment of *in vitro* cultures

First 3-4 passages were taken on MS medium fortified with 6 mg/l BAP + 20 mg/l Casein Hydrolysate + 30 mg/l $AdSO_4$ [23]. The multiplication medium which performed better in terms of healthy, long and good quality micro shoots during incubation period was selected for further experimental studies.

Methodology of gamma radiation treatment

Shoot cultures of fourth and fifth subculture were selected for gamma irradiation. These shoots were subcultured onto fresh medium and seven days later, these shoots were exposed to gamma irradiation using ^{60}Co of BRIT gamma chamber-5000. This research is attempt to test the results of gamma-ray irradiation treatment dose of 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 Gray and unirradiation (as a control) which aims to determine the characteristics of generative growth of red banana. Three replications were applied to each dose and each replication consisted of 20 shoots. Irradiation was done at Board of research in nuclear sciences, BARC, Mumbai. Culture bottle jars were labeled according to the dose treatment. Exposure time (in seconds) was calculated based on the dose rate (Gy/s) of the gamma source available in the gamma chamber (6.065 KGy/hr) on the day of carrying out the irradiation. After irradiation, these explants were transferred into similar fresh medium. Physiological and morphological observations were recorded. After each four weeks, subculture was carried out by transferring new grown shoots into fresh medium. After 4 weeks of each subculture, percent survival of plantlets were recorded and the effective dose level estimated (LD_{50} dose).

In vitro multiplication and rooting:

Unirradiated (control) and gamma irradiated cultures were transferred to multiplication medium for shoot multiplication. Subculturing of these shoots were carried out successively every 30 days up to four subculture cycles. After each subculture percent survival, length of shoots, number and length of leaves were measured.

Good quality microshoots (20–30 mm long) produced from *in vitro* multiplication stage was used for root induction. The individual shoot bases were cut transversely and five shoots were inoculated in each culture bottle jar. Individual shoots were transferred to full strength MS medium supplemented with IBA (2mg/l) and NAA (0.5mg/l). After rooting phase plantlets were taken out from culture bottle jars and washed thoroughly, to remove adhering agar medium to roots. The plantlets were treated with 0.2% carbendazim solution for 30 minutes. Later, these plantlets were transferred to a pro-tray containing coco-peat and FYM (1:1) and kept under greenhouse for 4 weeks. Plants were further transferred to polybag containing potting mixture of red soil + sand + FYM (1:1:1 v/v) and raised under shade house.

Culture conditions

During growth period, all *in vitro* cultures were kept under controlled conditions around 25±2°C temperature, light for 16 hr photoperiod under fluorescent light (2000 lux) and 8 hours in the dark with 55-60% relative humidity. Observations were recorded regularly in each week of culturing.

Data Collection and Statistical Analysis:
Analysis of variance, for effect of gamma irradiation on *in vitro* shoots of red banana were analyzed in completely randomized design (CRD). Values in percentage were subjected to angular transformations. Data collected in the CRD of experiments [25] was analyzed using OPSTAT (14.139.232.166/opstat/), CCS HAU, Hisar. Radio-sensitivity/ post-irradiation responses of *in vitro* cultures to various lethal dose values were recorded based on the 50% decrease in the survival rate of culture. If experimental treatments found significant, their relative performance was tested with critical difference ($CD_{0.05}$). The survival percentage of the gamma treated explants was calculated based on the formula:

$$\text{Survival Percentage (\%)} = \frac{\text{No. of explants survived after gamma exposure}}{\text{Total no. of explants irradiated with gamma ray}} \times 100$$

Mutant plant population (M_1V_5)
Total 5,000 plantlets were produced in two steps. In first step, approx. 3,200 plantlets were produced, from which total 2,185 well grown plantlets were transferred to field of Centre Research Station, Wakavli and DBSKKV University campus, Dapoli. Secondly, 1,800 plantlets generated from irradiated *in vitro* cultures from which 1,025 plants were planted successfully in Centre Research Station, Wakavli. These M_1V_5 plantlets were observed for their field performance and variant's evaluation. Form total plants, well developed 3,200 plants were used for further study.

DNA Isolation and Plant Selection
The extraction of genomic DNA followed the procedure described by Edwards *et al.,* (1991) [26]. Total 3,200 plants were chosen for the present experimental investigation. DNA was isolated in sample pooling method. 32 pooled DNA samples were obtained and each sample containing the DNA of 100 plants, which were then examined by PCR (Table-2). To evaluate the quantity and quality of DNA, comparisons were made by assessing band intensities on an agarose gel.
Dwarf-specific primers, derived from Ramage *et al*., (2004) [27], Damasco *et al.,* (1996) [28] and Suprasanna *et al.,* (2008) [29] utilized for selection of Dwarf plants from M_1V_5population (Table-3). The PCR mixture of 10 µl included the primers (1 µl each, 250 nM), Taq DNA polymerase (1 µl, 3 U/µl), 200µM of each dNTP (0.5 µl), $MgCl_2$ 25 mM 0.25 µl, Molecular Grade Water 5.75 µl, 10X PCR *Taq*. buffer 1.25 µl (10 mM Tris-HCl, 4.5 mM $MgCl_2$, 50 mM KCl, pH 8.3), and 20-25 ng of genomic DNA as the template. The thermocycler was programmed at 94 °C for an initial denaturation of 3 min, followed by 35 amplification cycles, each comprising steps at 94 °C for 10 Sec, (55 °C for B1 B2 & Dw1 Dw2, 50.2 °C for A1 A2) for 10 sec, and 72 °C for 60 Seconds, with a final extension for 5 min. The resulting amplified products were analyzed using agarose gel electrophoresis (1.4%). Gel visualization and photography were performed on a UV transilluminator after staining with ethidium bromide following the protocol by Sambrook *et al.,* (1989) [29]. The amplicon size of bands was measured by using UviTec software.

Results and Discussion
Determination of LD_{50} in Red banana:
Percent survival of irradiated material considered as one of the important criteria to estimate gamma dosage levels. The results shows significant difference in survival rate between the unirradiated (control = 0 Gray), low dosage and high dosage of gamma radiation exposure towards red banana *in vitro* culture. Results revealed that decrease of survival rate was observed with an increase in doses of gamma rays. Analysis on mortality of treated explants revealed that LD_{50} dose of gamma rays was optimum at 20 Gy for cultured shoot tips. Our results are supported by investigations of Mishra *et al* (2007) [30], who found that gamma radiation doses of 10 and 20 Gy induced good response for plantlet's development in three banana cultivars *viz*., Basari, Chakkarakela and Rasthali.
Theoretically, the highest frequency of mutations can be expected from a mutagen treatment killing about 50% of the treated materials (LD_{50}) and hence the LD_{50} dose of the gamma irradiation material shall be obtained from a radio-sensitive curve (Fig-1). Among the various irradiated treatments for *in vitro* cultures, the survival percent after 4 weeks was recorded from 37.33 percent in 30 Gy to 84.33 percent in 10 Gy as compared to the highest survival percent of 99.66 in unirradiated explants. The LD_{50} dose was found to be at 20 Gy based on its survival 57.66%. Doses beyond 40 Gy were completely lethal; all shoots turn brown and dead. Kulkarni *et. al.* (2004) [31] observed similar results in Grand naine banana, suggesting that higher doses more than 40 Gy proved to be lethal. The explants shows visible changes like browning from leaf margins, complete leaf browning to blackening, retardation of growth and eventually dead of shoots.
MS Medium supplemented with different concentration of BAP ranging from 1-8 mg/l. Maximum multiplication rate (1:1.8) of irradiated shoots were observed in medium fortified with 6 mg/l BAP + 20 mg/l casein hydrolysate + 30 mg/l Adenine sulphate ($AdSO_4$) with average shoot length of 2.66 cm. MS medium supplemented with 1.5 mg/l IBA and 0.5 mg/l NAA shows better results with 98.67% rooting. However, there was a significant difference in number of roots produced in irradiated shoots. The number of roots produced in irradiated shoots of 20 Gy was reduced as compaired to unirradiated shoots. This results shows that irradiation also affect on synthesis of auxin and/or cytokininin cultured plant cell/tissues.

Detection of Dwarf plants through Molecular markers:
Analysis was conducted on 3,200 selected red banana plants, isolating their tota DNA for subsequent screening. Employing a bulk analysis approach, these plant samples were organized into 32 pooled samples, as detailed in Table-2. Utilizing Polymerase Chain Reaction (PCR) with Sequence Characterized Amplified Region (SCAR) Primers, each pooled sample underwent scrutiny. Among 32 samples, only Sample No. 1 (which contains 100 plants) resulted a positive ("+"), signifying the presence of the dwarf gene, while the remaining all samples tested were negative ("-"). B1/B2 and Dw1/Dw2 primers shows Positive results, whereas A1/A2 primers yielded results that were challenging to interpret accurately, leading to their categorization as negative. This observation aligns with the study by Ramage *et. al*., (2004) [27], where B1/B2 SCAR primers demonstrated notable results in *in vitro* dwarf and wild-type banana leaf tissue, establishing consistency with the current findings.
Further study on positive Sample No. 1, comprising DNA from 100 plants done by sub-dividing this group into 10

sample lots, each containing DNA from 10 plants (Table 5), were conducted. From these sample lots, Sample No. 2, and Sample No. 6 gave positive results. Following positive results in Sample Groups 2 and 6, a more in-depth analysis was undertaken at the individual plant level, as elaborated in Table-6. The results revealed that plant No. 14, 18, and 52 showed positive results, suggesting the absence of specific genetic markers, while the other samples tested which are negative. In control and irradiated plants, we identified genes at 810 base pairs using SCAR (Dw1 and Dw2).

Discussion

Rate of multiple shoots formation were reduced with increase in dosage of gamma rays. Analysis on mortality of irradiated explants showed 20 Gy was LD_{50} dose optimum for *in vitro* cultures of red banana. Mishra *et al* (2007) [30] also reported 10Gy and 20Gy was optimum dose in Basari, Chakkarakela and Rasthali banana plantlets. High gamma radiation dosage prohibits regeneration of *in vitro* culture. Similar observation were recorded by various scientists [32, 30, 33, 34]. Gamma doses beyond 40 Gy were completely lethal to *in vitro* red banana cultures, all shoots turn brown and simultaneously dead. Kulkarni *et al* (2004) [31] observed similar results in Grand naine banana. Irradiated cultures shows physiological changes like browning in leaf margins and leaf browning. MS medium supplemented with BAP (6 mg/l), Casein hydrolysate (20 mg/l) and Adenine sulphate (30 mg/l) induces maximum micro-shoot formation (multiplication rate) (1:1.8) of irradiated shoots. MS medium supplemented with IBA (1.5 mg/l) and NAA (0.5 mg/l) shows better results, however, fluctuations in number of roots per shoots were observed. This result shows that irradiation also affect on synthesis of auxin and/or cytokininin cell/tissues. These findings are comparable with Karmarkar *et. al.* (2001) [35]; Kiong *et al.* (2008) [36] and Wi *et. al.* (2007) [37], statements that mutagens induces adverse effect on rooting.

The utilization of specific SCAR markers, including B1/B2 and Dw1/Dw2, has proven effective in identifying dwarf traits, aligning with similar studies done by Ramage *et al.,* (2004) [27] and Suprasanna *et al.,* 2008 [29].

Table 1: Surface sterilization of red banana (Sawardekar and Sherkar 2021) [23].

Sterilizing agents	Concentration of sterilizing agents	Time
Bavistin + Streptomycin	1 gm/l + 0.100 gm/l	30 min
Cefotaxime + Kanamycin	0.200 gm/l + 0.200 gm/l	30 min
Ethanol	70%	1 min
NaOCl	10%	10 min
$HgCl_2$	0.1%	5 min

Table 2: DNA Samples of Red Banana (Pooled sampling)

Sr. No.	No. of Pooled samples of DNA	Sample No.	Sr. No.	No. of Pooled samples of DNA	Sample No.
1.	1 – 100	1	17.	1601 – 1700	17
2.	101 – 200	2	18.	1701 – 1800	18
3.	201 – 300	3	19.	1801 – 1900	19
4.	301 – 400	4	20.	1901 – 2000	20
5.	401 – 500	5	21.	2001 – 2100	21
6.	501 – 600	6	22.	2101 – 2200	22
7.	601 – 700	7	23.	2201 – 2300	23
8.	701 – 800	8	24.	2301 – 2400	24
9.	801 – 900	9	25.	2401 – 2500	25
10.	901 – 1000	10	26.	2501 – 2600	26
11.	1001 – 1100	11	27.	2601 – 2700	27
12.	1101 – 1200	12	28.	2701 – 2800	28
13.	1201 – 1300	13	29.	2801 – 2900	29
14.	1301 – 1400	14	30.	2901 – 3000	30
15.	1401 – 1500	15	31.	3001 – 3100	31
16.	1501 – 1600	16	32.	3101 – 3200	32

Table 3: Primer List for Identification of Dwarf Type Mutants (SCAR Markers)

Sr. No.	Primer Name	Sequences	Annealing Temp	References
1	A1	5' CCG AAC ACG GGA CTT ATA CA 3'	50.2 °C	[27, 28]
2	A2	5' CCG AAC ACG GGC TAA CCT AG 3'	50.2 °C	
3	B1	5' CTG TGG TTG CAT TCT CAT AC 3'	55.0 °C	
4	B2	5' GTG AAT CAT ACT CGC GAA CC 3'	55.0 °C	
5	C1	5' TTA AGC CTT GGA TTG ACT GC 3'	55.0 °C	
6	DW1	5' CTG TGG TTG CAT TCT CAT AC 3'	55.0 °C	[29]
7	DW2	5' CTG AAT CAT ACT CGC GAA CC 3'	55.0 °C	

Table 4: Screening through PCR for Dwarf trait identification using SCAR Markers.

Sr. No	Sample No	No. of Pooled samples of DNA	SCAR Markers		
			A1 & A2	B1 & B2	Dw1 & Dw2
1.	1	1 – 100	-	+	+
2.	2	101 – 200	-	-	-
3.	3	201 – 300	-	-	-
4.	4	301 – 400	-	-	-
5.	5	401 – 500	-	-	-
6.	6	501 – 600	-	-	-
7.	7	601 – 700	-	-	-
8.	8	701 – 800	-	-	-
9.	9	801 – 900	-	-	-
10.	10	901 – 1000	-	-	-
11.	11	1001 – 1100	-	-	-
12.	12	1101 – 1200	-	-	-
13.	13	1201 – 1300	-	-	-
14.	14	1301 – 1400	-	-	-
15.	15	1401 – 1500	-	-	-
16.	16	1501 – 1600	-	-	-
17.	17	1601 – 1700	-	-	-
18.	18	1701 – 1800	-	-	-
19.	19	1801 – 1900	-	-	-
20.	20	1901 – 2000	-	-	-
21.	21	2001 – 2100	-	-	-
22.	22	2101 – 2200	-	-	-
23.	23	2201 – 2300	-	-	-
24.	24	2301 – 2400	-	-	-
25.	25	2401 – 2500	-	-	-
26.	26	2501 – 2600	-	-	-
27.	27	2601 – 2700	-	-	-
28.	28	2701 – 2800	-	-	-
29.	29	2801 – 2900	-	-	-
30.	30	2901 – 3000	-	-	-
31.	31	3001 – 3100	-	-	-
32.	32	3101 – 3200	-	-	-

"+" Indicates the presence of dwarf gene.
"-" Indicates the absence of dwarf gene.

Table 5: Sample lot No.-1: Showing Positive Result contains 100 plant DNA Samples (divided into 10 Samples Groups and each contains 10 plants DNA samples).

Sr. No.	Sample No.	Sample group	Result
1.	1	1-10	Negative
2.	2	11-20	**Positive**
3.	3	21-30	Negative
4.	4	31-40	Negative
5.	5	41-50	Negative
6.	6	51-60	**Positive**
7.	7	61-70	Negative
8.	8	71-80	Negative
9.	9	81-90	Negative
10.	10	91-100	Negative

Table 6: Results from sample lot 2 and sample lot 6.

Sr. No.	Sample No.	Result	Sr. no.	Sample No.	Result
1.	11	Negative	11.	51	Negative
2.	12	Negative	12.	52	Positive
3.	13	Negative	13.	53	Negative
4.	14	Positive	14.	54	Negative
5.	15	Negative	15.	55	Negative
6.	16	Negative	16.	56	Negative
7.	17	Negative	17.	57	Negative
8.	18	Positive	18.	58	Negative
9.	19	Negative	19.	59	Negative
10.	20	Negative	20.	60	Negative

Table 7: Distinctive Features of Red Banana unirradiated and Irradiated Red Banana plants

Plant No.	Days for flowering	Plant Height (Cm)	Pseudostem Diameter (Cm)	Hands/ Bunch	Total Fingers/ Bunch	Avg. No of fingers/ Hand
C-261	290	380	28.63	5	78	15.60
C-206	290	354	25.45	6	92	15.33
C-158	273	337	29.27	6	109	18.16
C-205	242	335	30.22	6	105	17.50
C-202	245	333	28.63	5	81	16.20
Avg. control plants	268	347.8	28.44	5.6	93	16.558
Identified dwarf plants(irradiated):						
I-18	234	296	83	26.43	5	77
I-14	239	287	78	24.84	5	66
I-52	278	282	75	23.88	5	68
Other plants (irradiated)						
I-133	305	298	22.27	4	44	11.00
I-22	308	291	21.00	4	34	8.5
I-345	285	303	23.86	4	40	10.00
I-323	288	304	27.04	5	82	16.40

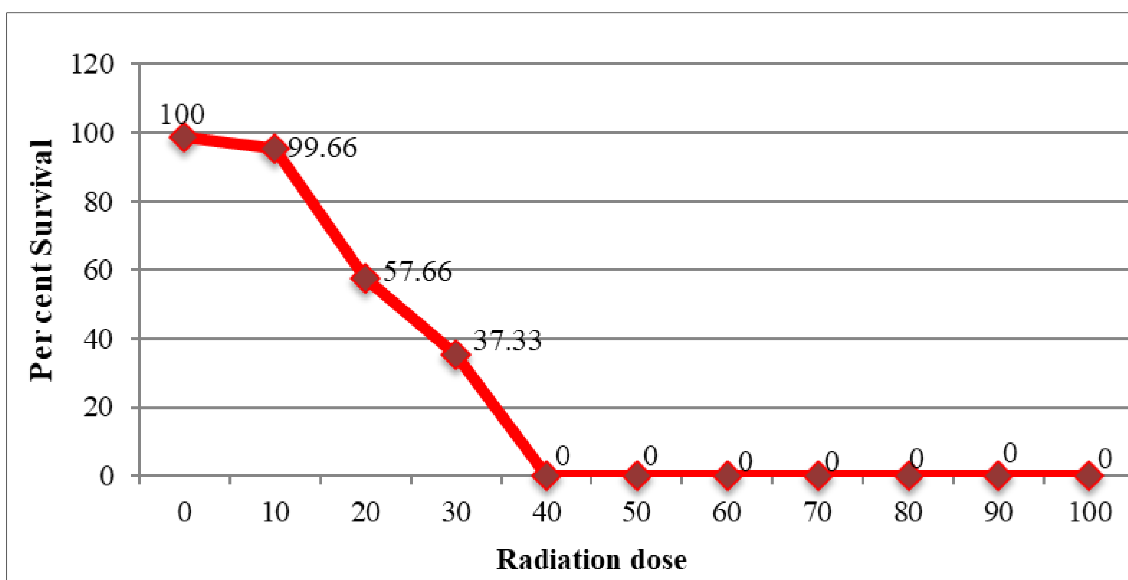

Fig 1: Radio sensitivity test curve: Percent survival of plants.

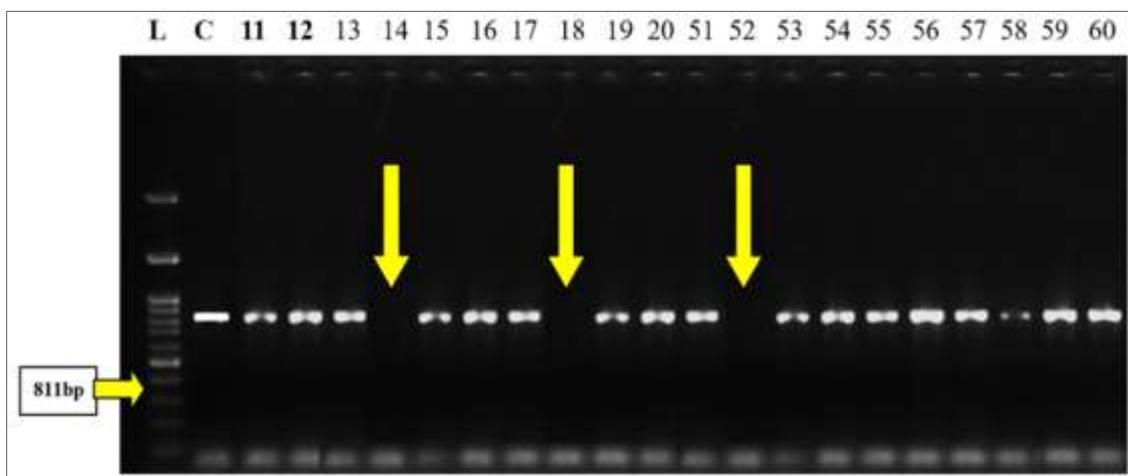

Fig 2: Identification of dwarf red banana plants by using SCAR (Dw1 & Dw2) marker (811 bp). Sample 14, 18 and 52 showing Positive result. Plant no's 11 – 13, 15-17, 51, and 53-60 inducting Tall plants.

Fig 3: M_1V_5 population at Dr. BSKKV, Dapoli, Ratnagiri, Maharashtra.

A B

Fig 4: Comparison between A-Control (unirradiated) and B-irradiated plants.

Conclusions

In conclusion, the outcome of the present study indicated that gamma dosage beyond 40 Gy gave negative effect on growth which shows 100 percent mortality in irradiated cultures of red banana. During *in vitro* passages, the most appropriate LD_{50} dose of gamma irradiation for inducing growth and morphological variation in red banana was found to be 20 Gy (57.66% survival rate). Also, the study has shed light on the genetic markers associated with dwarfism in red bananas. Utilizing molecular markers such as SCAR Primer (Dw1 & Dw2 primers has provided an extensive genetic profile of these red banana anomalies. The positive findings in Samples No./plant No. 14, 18, and 52 that exhibit specific genetic markers signify promising candidates for future study. Considering these findings, the thorough molecular screening serves to illuminate the wider implications for breeding programs as well as the genetic characteristics associated with dwarfism in red banana mutants. This investigation useful in utilizing gamma irradiation to *in vitro* cultures in red banana for selection of desirable mutants.

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