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Characterization and evaluation of putative mutant populations of polyembryonic mango genotype Nekkare for dwarfing rootstock traits

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ABSTRACT

Availability of dwarfing rootstocks is an important pre-requisite for improving productivity of mango orchards in India as it facilitates high density planting as well as impart uniformity within an orchard. An attempt was made to induce variability in polyembryonic mango genotype Nekkare for dwarfness by treating kernels with different doses of gamma radiation ranging from 15 to 35 Gy. Irradiation created significant variation in plant height, stem girth, number of nodes, inter-nodal length, number of leaves, leaf blade length and leaf blade width. The highest reduction in seedling height along with highest variation was observed at 35 Gy where the seedling height ranged from 11.50 to 33 cm with a mean of 23.12 cm as compared to mean plant height of 44.55 cm in control ranging from 33.50 to 56 cm. Further, the effect of irradiation on stomatal parameters was also investigated and the highest stomatal length and width was recorded at 15 Gy (63.39 µm) and 20 Gy (63.12 µm) respectively while 30 Gy treatment produced maximum stomatal density (13.85 per µm^2). Furthermore, the concentration of ABA was found to be highest (429.1 ng/gm) in morphologically dwarf (putative mutant) progenies of Nekkare. The results suggest effectiveness of induced mutation for developing dwarfing rootstocks in mango to be used in high density planting.

Keywords: Gamma irradiation, mango, mutation, Nekkare and stomatal parameters

INTRODUCTION

Mango (*Mangifera indica*) enthroned as the king of fruits in India enjoys a eminent position in the horticultural bounty of the country. Indian mango industry has a global presence in production as well as export of fresh fruits and processed products. India is the largest producer of mango in the world accounting for more than 55% of total world's production with an area of 2,293,000 ha and production of 20,798,000 MT (NHB, 2019). However, the productivity (per hectare yield) of mango in the country remains as low as 8 t/ha. Low planting densities, presence of old and senile orchards, propagation on seedling rootstocks of unknown origin, irregular bearing habit and lack of genetically uniform dwarfing rootstocks are considered as some of the major reasons for low orchard efficiency of mangoes in India. In the purview of global climate change and need for intensive cropping to meet the domestic as well as export demand, potential of polyembryonic genotypes to be used as rootstock needs to be properly investigated. Meanwhile, the lack of genetic variability in these genotypes owing to the presence of true to type nucellar seedlings limits their use in the mango rootstock breeding programme. Mutation breeding has been extensively used in several crops for improvement of yield or yield attributing traits as well as for enrichment of existing germplasm (Ahloowalia and Maluszynski, 2001). In heterozygous crops like mango also, induced mutations have been widely used for creation of variability for different traits (Karsinah *et al.,* 2012; Rime *et al.* 2019). In mutation breeding, gamma rays and ethyl methane sulfonate (EMS) are the most commonly used physical and chemical mutagens respectively. Induced mutation has been known to create variations in plant height, growth pattern, leaf characteristics and various biological and

physiological traits including stomatal size and number (Kovacs and Keresztes, 2002). Stomatal structure (including size and density) is the major plant cellular component contributing towards growth and development (owing to its role in gaseous exchange and transpiration thus directly affecting photosynthesis) of the plants besides playing a crucial role in stress tolerance (Yasmeen *et al.*, 2020). Hence, any change in stomatal parameters resulting from mutagenesis could be highly significant. Here, an attempt was made to create variability in a vigorous polyembryonic mango genotype Nekkare, known for its tolerance to salinity stress (Pandey *et al.*, 2014; Laxmi *et al.*, 2021) for plant stature employing induced mutation approach using gamma irradiation. The objective of this study was to determine the variability created among the selected putative mutants generated through different doses of gamma irradiation ranging from 15 Gy to 35 Gy, for various morphological traits as well as to investigate the structural changes in stomatal parameters followed by hormonal profiling of selected putative mutants to understand the effectiveness of induced mutation for developing dwarfing rootstocks in mango to be used in high density planting.

MATERIALS AND METHODS

Site of experiment and plant material

This research was conducted in ICAR-Indian Institute of Horticultural Research, Bengaluru. Six month old putative mutant seedlings of polyembryonic mango variety Nekkare used in this study were generated by treating seed kernels of Nekkare with five different doses of gamma irradiation viz., 15Gy, 20Gy, 25Gy, 30Gy and 35Gy (280 kernels per treatment) (unpublished data). From the putative mutant population thus generated, a total of 100 putative mutant seedlings (20 from each treatment) along with control (untreated nucellar seedlings) of short, medium and tall stature (based on plant height) were selected for taking further observations.

Morphological characterization

Morphological observations on plant height (cm), stem girth (mm), number of internodes, inter-nodal length (cm), number of leaves, leaf blade length and leaf blade width for 100 selected putative mutant progenies of Nekkare along with control were recorded based on mango descriptor (IPGRI 2006). Volatile profiling was used to ascertain the nucellar origin of control seedlings (unpublished data).

Stomatal parameters

Again, for these 100 putative mutants and control seedlings, stomatal size and number was determined using the procedure as described by Hamil *et al.* (1992). For measuring number of stomata, fully matured and expanded leaves were selected and plucked between 12-01 PM. Freshly plucked leaves were kept in butter paper, labelled properly and brought to laboratory for further analysis. For determining the number of stomata, abaxial surface of fully matured and expanded leaves plucked between 12 noon to 1 PM coated with transparent nail polish and left for 30 minutes to dry. Glass slides were prepared by peeling off the imprinted layer from the abaxial surface of the leaf sample. The slides were then examined under the microscope (OLYMPUS; Light microscope digital camera; DP-22/DP-27) at a magnification of 10x and stomata numbers were recorded and expressed as number of stomata per mm^2. For measuring stomatal size, slides were examined at a magnification of 40x and length and width of stomata was recorded and expressed in µm.

Phytohormone profiling

Further, morphologically distinct dwarf and tall statured putative mutant seedlings along with control plants were selected for phytohormone profiling using Liquid chromatography-mass spectrometry (LC-MS).

Extraction procedure

Phyto-hormones were extracted using the method described by Pan *et al.* (2008). For extraction, 3g leaf sample was completely homogenized using 1-propanol/H_2O/concentrated HCl (2:1:0.002, v/v/v) followed by sonication for 30 minutes at 4°C keep and kept overnight. Next day dichloromethane was added to the homogenate and sonicated for 30 min followed by centrifugation at 12,000 rpm for 10 minutes. After centrifugation, the bottom layer was transferred to a conical flask containing sodium sulphate to remove any traces of water and then evaporated using flash evaporator. Once the sample was completely dried, it was dissolved in 80% methanol and loaded through C18 solid-phase extraction (SPE) cartridges.

The SPE process included: (a) Pre-conditioning: It included washing the column with 5 mL methanol and 5 mL 80% methanol. (b) Adsorption: After the extract

was loaded to the column, all matrices were adsorbed by the cartridge and (c) Elution: In this step, the residual plant hormones were eluted with another 5 mL 80% methanol.

The obtained elute (5 mL) was then evaporated using flash evaporator at 35°C and finally the dried sample was dissolved in 500 µL methanol–0.05% formic acid (1:1, v/v). The solution was filtered using a nylon filter paper and injected into LCMS for further analysis.

LC and MS-MS conditions

The initial gradient was composed of 85% solvent A and 15% of solvent B with a hold time of 1 min. At 12 minutes, the gradient was changed to 15% of solvent A and 85% of solvent B, with a hold time for 1 minute and at 14 minutes, linear gradient was followed by 85% of solvent A and 15% of solvent B with a hold time of 0.5 minute. The system was then returned to the initial conditions at 15 mins and equilibrated for 1 minute before the next injection. The flow rate was 0.2 mL/minute. The analytical column was 2.1 x 50 mm UPLC BEH-C18 column (Waters, USA) with 1.7µm particles, protected by a vanguard 2.1 X 5 mm BEH C-18 with 1.7µm. Guard column (Waters, USA) was used with column temperature maintained at 25°C. Identification and quantification of the elute hormones was done using a TQDMS/ MS (Waters, USA) system, optimized for the hormone analysis.

Mobile phase used were

(i) Solvent - A: water/Acetonitrile/acetic acid (95/5/ 0.05, v/v/v) and (ii) Solvent - B: Acetonitrile/water/ acetic acid (95/5/0.05, v/v/v)

Statistical analysis

Descriptive statistics was calculated for morphological and stomatal parameters using standard procedure.

RESULTS AND DISCUSSION

Effect of gamma irradiation on morphological traits

Gamma rays are the most widely used physical mutagen employed in mutation breeding of crop plants and are well known for bringing about morphogenetic and endomorphic changes in plants (Ali *et al.*, 2016; Yasmeen *et al.*, 2020). In the present study considerable variation was created for different morphological traits in the putative mutant population of polyembryonic mango genotype Nekkare generated by gamma irradiation (Table 1a and 1b; Fig 1a-f). Plant height was found to be the most affected trait by irradiation (Fig. 2 and 3). A reduction in plant height (in comparison to control) with increasing dosage of irradiation was recorded with highest mean plant height in control being 44.55 cm while among the treatments, mean plant height ranged from 28 cm (15 Gy) to 21.35 (25 Gy). Further, although the mean plant height of population generated through 35 Gy treatment was 23.12 cm, the progenies it produced were as short as 11.50 cm and the maximum plant height recorded in this treatment was 33 cm in contrast to control where the plant height ranged from 33.50 to 56 cm. The use of gamma irradiation for developing dwarf statured mango varieties to be used for high density planting has been attempted for a very long time (Sharma and Majumder, 1985, 88; Sharma, 1987).

A decrease in stem girth with increasing doses of irradiation was also observed and the highest mean stem girth was recorded in control (9.07 cm) which declined to 6.53 cm in 25 Gy treatment. However, the progeny with largest stem girth (11.32 cm) was produced by 35 Gy treatment. Mutation affects the process of cell division and cell elongation along with disruption of protein synthesis, hormonal and enzymatic balance and water and gaseous exchange ability of plants thus leading to reduction in plant height as well as overall growth of plants (Ali *et al.*, 2016; Asare *et al.*, 2017). The results of the present study are in confirmation with earlier findings reporting the reduction in plant height with increasing doses of irradiation in mango (Rime *et al.* 2019), guava (Zamir *et al.*, 2003), grapes (Surakshitha *et al.*, 2017) and apple (Atay *et al.*, 19).

Further, the variation for number of nodes was highest in control along with highest mean number of nodes being 7.2 while the lowest mean number of nodes was recorded in 15 Gy being 5.76. Mean inter-nodal length also declined with increasing doses of irradiation. The number of leaves in all the treated populations was less than control where the mean number of leaves was 24.60 ranging from 17 to 35. Among the treated populations, the lowermost treatment (15 Gy) produced highest mean number of leaves ranging from 9 to 35 while the lowest mean number of leaves (15.93). Reduction in number of leaves with increasing doses of gamma irradiation has been reported in Arumanis mango of Indonesia (Karsinah *et al.,* 2012) and EMS derived population of mango hybrid Arka Puneet (Rime *et al.,* 2019).

Gamma irradiation resulted in the production of smaller and narrower leaves exhibiting a reduction in leaf blade length with increasing dosage of irradiation (Fig. 4). The highest mean blade length was recorded in control (18.15 cm) which decreased to 11.62 cm in 35 Gy treatment while the lowest leaf blade length (11.21 cm) with highest CV (17.13) was observed in 25 Gy treatment. Following the same trend, the mean leaf width was found to be highest in control being 5.56 cm which decreased in the entire gamma irradiated population exhibiting highly profound reduction in the higher dosage of irradiation and the lowest leaf blade width was recorded for 35 Gy treatment being 3.65 cm along with highest CV (14.23). In contrary to our results, Rime *et al.,* (2019) reported production of longer and wider leaves in the EMS derived putative mutant population of mango hybrid Arka Puneet while it is in congruence with the findings of Surakshitha *et al*. (2017) where they observed a gradual reduction in leaf length and leaf width with increased dosage of gamma rays in grape cultivars Red Globe and Muscat.

1a : Nekkare Control

1b : Treatment 1 (15 Gy)

1c : Treatment 2 (20 Gy)

1d : Treatment 3 (25 Gy)

1e : Treatment 4 (30 Gy)

1f : Treatment 5 (35 Gy)

Fig. 1 : Seedlings of Nekkere plants raised from gamma irradiated seeds

Fig. 2 : Dwarf putative mutant of Nekkare

Fig. 3 : Dwarf putative mutant of Nekkare

Fig. 4 : Narrow leaf shape in Nekkare mutant

Table 1a : Morphological variability induced in polyembryonic mango variety Nekkare through different doses of gamma irradiation.

Treatment	Plant height (cm)				Stem girth (cm)				Number of nodes				Inter-nodal length (cm)			
	Mean	Min	Max	C.V.	Mean	Min	Max	C.V.	Mean	Min	Max	C.V.	Mean	Min	Max	C.V.
Control	44.55	33.5	56	16.41	9.07	8.22	10.02	7.5	7.2	5	12	29.86	6.1	3	9	28.08
15 Gy	28	14	41.5	30.86	7.64	4.15	10.94	26.97	5.76	4	10	26.4	4.5	2	8	32.87
20 Gy	24.47	16	36	21.49	7.41	4.87	10.22	16.88	6.19	4	8	20.63	3.75	2	6	27.54
25 Gy	21.35	12.5	36.5	26.66	6.53	3.6	9.13	22.21	6.47	5	9	14.16	3.27	2	6	32.95
30 Gy	22.87	16	35.5	21.62	7.18	5.06	10.89	22.57	6.42	4	10	25.6	3.58	2.5	5	22.9
35 Gy	23.12	11.5	33	32.75	7.34	5.22	11.32	24.71	5.85	4	8	18.27	3.62	2.5	6.5	29.96

Table 1b : Morphological variability induced in polyembryonic mango variety Nekkare through different doses of gamma irradiation

Treatment	Number of leaves				Leaf blade length (cm)				Leaf blade width (cm)			
	Mean	Min	Max	C.V.	Mean	Min	Max	C.V.	Mean	Min	Max	C.V.
Control	24.6	17	35	23.17	18.15	13.54	21.16	13.01	5.56	4.6	6.76	11.77
15 Gy	18.76	9	35	39.23	13.43	7.42	15.04	13.07	4.08	3.28	5.1	11.87
20 Gy	17.94	9	31	28.89	11.63	9.06	14.8	15.08	3.88	3.18	4.64	10.11
25 Gy	15.93	9	29	43.06	11.21	8	14	17.13	4.03	3.36	4.74	10.8
30 Gy	18.21	9	31	33.58	11.94	9.8	16.3	15.13	3.73	3	4.54	8.84
35 Gy	18.92	9	30	34.82	11.62	8.6	15	16.93	3.65	2.66	4.56	14.23

Stomatal parameters

In our study we observed high variability for different stomatal parameters like stomatal density, length and width of stomata (Table 2; Fig. 5a-d). Highest mean stomatal length (65.07 µm) was recorded in control (64.55 to 65.43 µm) with a CV of 0.65. Among the putative mutant populations, the highest (63.39 µm) and lowest (61.9 µm) mean stomatal length was recorded in 15 Gy and 35 Gy irradiation treatments respectively (Table 2).

Variations in stomatal width was also recorded in the putative mutant populations and the mean stomatal width of all the treatments except the putative mutant population generated through 25 Gy irradiation was found to be higher than control plants which showed less variation for this trait having a CV of 0.99 and mean stomatal width of 60.83 µm varying between 60.15 to 61.57 µm. Among the putative mutant populations, the mean stomatal width increased at lower doses (15 and 20 Gy) while it declined at higher doses (30 and 35 Gy) while remaining higher than control. The highest (63.12 µm) and lowest (60.92 µm) mean stomatal width was recorded in population generated through 20 Gy and 25 Gy respectively. An increase in stomatal length and width was recorded in sugarcane at lower doses of gamma irradiation (10 and 20 Gy) which declined further with increased doses (30 and 40 Gy) (Yasmeen *et al.* 2020). Similarly, a significant reduction in stomata length and number at higher doses of gamma irradiation was observed in Kinnow (Mallick *et al.,* 2016), grapes (Kok, 2011) and mango (Rime *et al.*, 2019).

Stomatal density was found to decrease with increasing dosage of irradiation and a profound variation was observed among the populations developed by different treatments as revealed by the values of coefficient of variability. The mean stomatal density for control plants was recorded to be 14.33 (per 20000 μm^2) with a very low coefficient of variability being 4.94. Among the treatments, mean stomatal density decreased in lower doses viz., 15 Gy and 20 Gy of being 12.64 (per 20000 μm^2) and 12.43 (per 20000 μm^2) respectively while it increased at 25 Gy (13.3 per 20000 μm^2) and 30 Gy (13.85 per 20000 μm^2). Our results are in contrary to the findings of Yasmeen *et al.* (2020) who reported an increase in stomatal density at lower doses of irradiation. However, the highest dose of irradiation (35 Gy) resulted in marked reduction in the stomatal density being 11.82 (per 20000 μm^2). A lower stomatal density is indicative reduced plant vigour and stature (Majumder *et al.*, 1981) and this result is also confirmed by the production of highly reduced plant height at 35 Gy treatment in this study. Dwarfing pear variety, '601D' is reported to have shorter intermodal length, thick and broad leaves, lower stomatal density and larger stomatal size as compared to it vigorous mutant '601T' (Liu *et al.*, 2022). The decline in stomatal number at higher doses of irradiation has also been reported in citrus (Mallick *et al.*, 2016), mango (Rime *et al.*, 2019) and sugarcane (Yasmeen *et al.*, 2020).

Correlation analysis

Correlation analysis of physical mutagenesis with the stomatal and plant morphological parameters gave interesting insights into the data (Table 3). In general, the overall correlation between doses of gamma irradiation and stomatal (except stomatal width) and plant morphological parameters seemed to be negative and traits like plant height, stem girth, leaf blade length, leaf blade width and stomatal length showed highly strong negative correlation with irradiation doses (-0.899, -0.815, -0.887, -0.914 and -0.919 respectively). Contrarily, a strong positive correlation was observed between stomatal length and morphological traits viz., plant height, stem girth, number of leaves, leaf blade length and leaf blade width (0.919, 0.902, 0.843, 0.907 and 0.875 respectively). Further, a positive correlation between stomatal length and stomatal density was seen (0.611) while stomatal width was negatively correlated with stomatal density (-0.600). Different morphological traits like plant height, stem girth, number of leaves, leaf blade length, leaf blade width divulged positive association among each other and significant positive correlations were observed between plant height and stem girth (0.961), number of leaves (0.957), leaf blade length (0.994), leaf blade width (0.964). A positive correlation between stomatal density and tree height has been reported previously (Camargo and Marenco, 2011, Yasmeen *et al.,* 2020) and increased stomatal density has been found associated with reduced tree height (Barrientos-Pérez and Sánchez-Colín, 1982).

Table 2 : Variability in stomatal density, stomatal length and width induced in polyembryonic mango variety Nekkare through different doses of gamma irradiation

Treatment	Stomatal density (per 20000 µm area)					Stomatal length (µm)					Stomatal width (µm)				
	Mean	St. Err.	Min	Max	CV	Mean	St. Err.	Min	Max	CV	Mean	St. Err.	Min	Max	CV
Control	14.33	0.53	12.67	17.33	4.94	65.07	0.19	64.55	65.43	0.65	60.83	0.27	60.15	61.57	0.99
15 Gy	12.64	0.48	9	18	18.98	63.39	0.42	59.09	68.03	3.34	62.87	0.47	58.41	69.7	3.73
20 Gy	12.43	0.58	7.33	18	22.52	63.34	0.7	55.39	69.95	5.28	63.12	0.51	57.05	68.65	3.86
25 Gy	13.3	0.51	9	17.67	16.69	62.13	0.63	56.86	66.11	4.42	60.92	0.52	55.61	63.57	3.72
30 Gy	13.85	0.36	8.67	15	12.84	62.82	0.73	57.62	70.76	5.06	61.43	0.55	56.02	65.1	3.92
35 Gy	11.82	0.67	10	17.67	18.77	61.9	0.74	56.86	66.11	4.48	61.59	0.58	55.61	64.96	3.53

5a : Nekkare Mother Plant

5b : Nekkare Control plant

5c Putative mutant seedling N 63

5d Putative mutant seedling N 91

Fig. 5 : Stomata character of Nekkare plants subjected to gamma irradiation.

Table 3 : Correlation analysis of doses of gamma radiation, stomatal parameters, morphological traits

Variables	Gamma irradiation doses	PH	SG	NN	NL	LBL	LBW	SD	SL	SW
Gamma irradiation doses	1									
PH	-0.899	1								
SG	-0.815	0.961	1							
NN	-0.620	0.696	0.552	1						
NL	-0.748	0.957	0.982	0.618	1					
LBL	-0.887	0.994	0.949	0.690	0.951	1				
LBW	-0.914	0.964	0.857	0.808	0.865	0.958	1			
SD	-0.563	0.579	0.431	0.872	0.483	0.619	0.677	1		
SL	-0.941	0.919	0.902	0.651	0.843	0.907	0.875	0.611	1	
SW	0.047	-0.281	-0.118	-0.665	-0.286	-0.317	-0.414	-0.600	-0.013	1

Values in bold are different from 0 with a significance level alpha=0.05
PH-Plant height; SG-Stem girth; NN-Number of nodes; NL-Number of leaves; LBL-Leaf blade length; LBW-Leaf blade width; SD-Stomatal density; SL-Stomatal length; SW-Stomatal width

Plant hormones

Cell elongation and proliferation are the key processes determining height of a plant and plant hormones regulate these processes in a species dependent manner (Yue *et al.*, 2016). Among the putative mutant population created by different dosage of gamma radiation 10 morphologically distinct dwarf and tall seedlings were selected to determine the levels of two growth promoting (IAA and GA7) and one growth inhibiting (ABA) phytohormones. IAA has been found to be associated with cell division, expansion and differentiation thus regulating the plant height (Liu *et al.*, 2022). GA is another important phytohormone that regulates plant height and most of the dwarf phenotypes are results of disruption in GA biosynthesis or signal transduction (Magome *et al.*, 2010). Nekkare is a vigorous genotype and the concentration of IAA was recorded to be highest (4.16 ng/gm) in control samples and tall (putative mutant) progenies (1.56 ng/gm) while dwarf (putative mutant) progenies were found to contain the lowest (0.78 ng/gm) amount of IAA. Similar to IAA, the highest (0.26 ng/gm) concentration of GA_7 was recorded in control followed by tall (putative mutant) progenies while the lowest amount of GA_7 occurred in dwarf (putative mutant) progenies (Table 4). The interaction between auxin and gibberellin has been known to have implications on plant growth where auxins act as a messenger compound that links the apical bud with biosynthesis of active GAs in the expanding inter-nodes (Ross and O'Neill, 2001). This suggests the possibility that the main growth-regulating function of endogenous IAA could be to maintain the levels of GA in the internode (Ross *et al.*, 2001). This relationship between IAA and GA was also observed in our study where the high levels of IAA in tall (putative mutant progenies) corresponded to the increased levels of GA_7 in the same. Great variation for the concentration of ABA was also recorded in the studied samples which was found to occur in concentration as high as 429.1 ng/gm in dwarf (putative mutant) progenies to as low as 47.61 ng/gm in control plants. Dwarfing apple and citrus trees are reported to contain more bark ABA concentration than the vigorous trees and exogenous application of ABA has resulted in shortened inter-nodes and decreased growth in apple (Noda *et al.* 2000; Tworkoski and Fazio 2011). Dwarf stature of pear variety '601D' is also considered to be a result of over accumulation of ABA (Liu *et al.*, 2022). The highest concentration of ABA in dwarf (putative mutant) progenies in our study further endorses the role of ABA in regulating plant height.

Table 4 : Concentration of plant hormones in control, tall and dwarf plants of polyembryonic mango variety Nekkare

Phytohomone (ng/gm)	Control	Tall	Dwarf
IAA	4.16	1.56	0.78
ABA	47.61	305.93	429.10
GA7	0.26	0.13	0.09

CONCLUSION

The results indicate that gamma irradiation is a potential tool for creating variability for morphological and endomorphic traits. Considerable variation for plant height and other morphological traits governing overall plant stature like inter-nodal length, number of leaves, leaf size etc. were recorded along with alteration in stomatal length, width and density. Reduction in plant height, shorter inter-nodes along with production of smaller and narrower leaves at higher dose of gamma irradiation (35 Gy) corresponded with occurrence of smaller and broader stomata with reduced stomatal density. Furthermore, the concentration of ABA was found to be highest in morphologically dwarf (putative mutant) progenies of Nekkare. The results confirm the effectiveness of higher dosage of gamma irradiation (35 Gy) for developing dwarfing rootstocks in mango to be used in high density planting.

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