Creation of variability by *in vitro* mutagenesis in cassava (*Manihot esculenta*, Crantz)

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Abstract

In vitro mutagenesis in cassava was carried out in the variety Sree Jaya in Department of Plant Breeding and Genetics, College of Horticulture, Kerala Agricultural University during 2012-2015. Sree Jaya is a high yielding variety of cassava developed by selection at CTCRI Trivandrum. It has six month duration and tuber yield of 26-30 Mg ha^{-1} with 24 -27 per cent starch. Somatic embryos and somatic embryo derived plantlets were exposed to EMS and Gamma ray. EMS was tried at concentrations of 0.3 to 1.2 per cent at an interval of 0.3 per cent. The cultures were irradiated with gamma rays at doses ranging from 10Gy to 80Gy with a dose interval of 10Gy. Immediately after the treatment the cultures were sub cultured in to fresh medium for regeneration. Based on the survival rate of the cultures the LD 50 values were fixed. For somatic embryos the LD 50 value was 30Gy for gamma radiation and 1.20 per cent for EMS. For somatic embryo derived plantlets LD 50 value was fixed at 50Gy for irradiation and 0.90 per cent for EMS. Significant difference in the growth characteristics of the mutagen treated *in vitro* cultures were observed with respect to quantitative traits like plant height, number of shoots and leaves at tissue culture and hardening stages. The qualitative traits like colour of the petiole, stipule, emerging leaf and stem varied among the mutated plants.

Key words: Cassava, Sree Jaya, *In vitro* mutagenesis, LD 50, Variation in qualitative traits

Introduction

Cassava (*Manihot esculenta* Crantz) is one of the most important carbohydrate source in the world and second important staple food in Kerala. *In vitro* mutagenesis has been identified as a potential tool to create genetic variability for cassava improvement because it gives faster results compared to true seed breeding approach, generates large genetic variability and increases the chance of successful selection for new cassava clones (Taylor et al., 1996). Mutation breeding approach has been practiced in India by Central Tuber Crops Research Institute (CTCRI) for development of varieties with cassava mosaic disease resistance and low level of HCN in the root (Abraham et al., 2002).

The present work was carried out with the objective of inducing variability through *in vitro* mutagenesis in cassava genotype Sree Jaya and evaluation of mutated population for quantitative and qualitative traits. Sree Jaya has been identified as a high yielding short duration variety in several trials along the Kerala state, its tuber fresh yield ranges between 26 and 35 Mg ha^{-1} when harvested at six months after planting (CTCRI, 2006, Suja et al., 2010 and Magaia et al., 2014).

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Materials and Methods

In vitro cultures of the genotype Sree Jaya was treated with physical and chemical mutagen gamma radiation and Eyhyl methyl suphonate (EMS), respectively. Somatic embryos (SE) at globular stage and plantlets with shoots of 0.5-1 cm length in seven tubes each were used for mutagen treatment. The Gamma rays exposure varied from 10 to 110Gy (at 10Gy intervals) and the concentration of EMS incorporated before autoclaving into solid media varied from 0.30% to 1.50% at an interval of 0.30% (Novak and Micke, 1988). EMS treated cultures were sub-cultured 72 hours after treatment. In order to perform *in vitro* gamma irradiation, the respective cultures were inoculated into small test tubes of 25x100 mm size and 45 ml capacity with 15 ml corresponding MS solid media. The culture tubes were organized in bunches of seven and tied with rubber bands for each treatment. Soon after the treatment the subculturing was carried out into fresh media. Evaluation of the effect of EMS on *in vitro* cultures of cassava was done by taking observations twice a week and recorded as number of survival of inoculated cultures over total number of cultures treated. The estimation of the respective LD50 was done for both Gamma-rays and EMS based on survival of the treated cultures.

In vitro regenerated plantlets of genotype Sree Jaya under different mutagenic treatments were evaluated, after seven weeks of culture, just before the planting out, for plant height (cm), number of shoots per culture, number of leaves per culture, number of roots per culture, length of roots per culture (cm) and size of the roots (score: 1 to 10). *In vitro* regenerated plantlets in cassava having at least 2.0 - 4.0 cm of height and with roots of 3-5 number, as minimal growth parameters (Jorge et al., 2000), were shifted to pad and fan green house (Dumet et al., 2007).

Once plantlets reach 30 cm height, application of nutrients was done with commercial preparation Greencare™ (N-P-K: 30-10-10, *i.e*., primary, secondary macro and micro nutrients) at 0.5% concentration gradually increasing the frequency of application. In the first week it was applied once in a week, and during second and third week thrice a week and from fourth week onwards the nutrient was applied on a daily basis. Similarly, plantlets of more than 30 cm height were gradually exposed to sunlight as follows: first week - one hour at 8-9 am; second week –two hours at 8-10 am; third week three hours at 8-11 am and fourth week - four hours from 8 am to 12 noon

Results and Discussion

LD_{50} value for gamma irradiation for somatic embryos and somatic embryo derived plantlets was fixed as 40Gy and 50Gy, respectively (Figure 1). The results suggested that somatic embryos were more sensitive to the intensity of gamma rays. On the other hand, the LD_{50} value of EMS for plantlets (0.90%) was lower than the value of somatic embryos (1.2%). Hence, results indicated that for the effectiveness of mutagenesis work, care should be taken to subject the plantlets to lower concentration of EMS than somatic embryos. The EMS treatment of 0.9 per cent for somatic embryo derived plantlets resulted in mortality of about half of the individual cultures. It resulted in 59 per cent survival of plantlets. However, treatment of 1.20% EMS resulted in survival of about half of the individual cultures of somatic embryos (Figure 2).

Evaluation of in vitro variability

In vitro techniques are being used effectively for induction of variability in many crops . Heat tolerant mutants in potato by *in vitro* mutation methods are produced (Das et al., 2000). Late blight resistant mutants were produced in potato using gamma rays (Aparna et al., 1999) . Chemical and irradiation induced mutation in *in vitro* culture has been successfully used to improve banana and plantain (Novak and Micke, 1988).

Visual examination of tissue culture plants *in vitro*

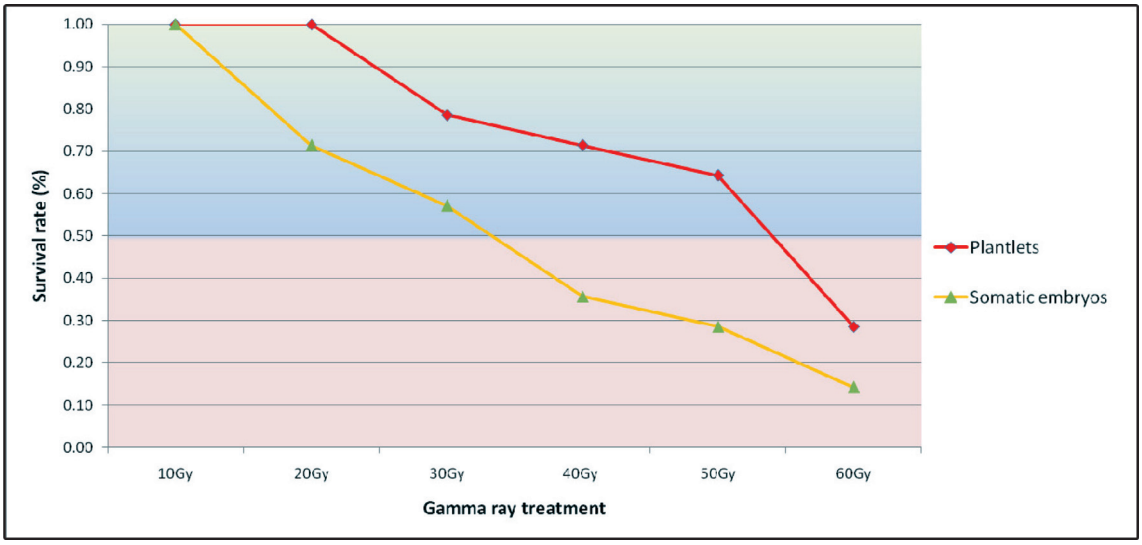

Figure 1. Response of cultures to gamma rays in cassava

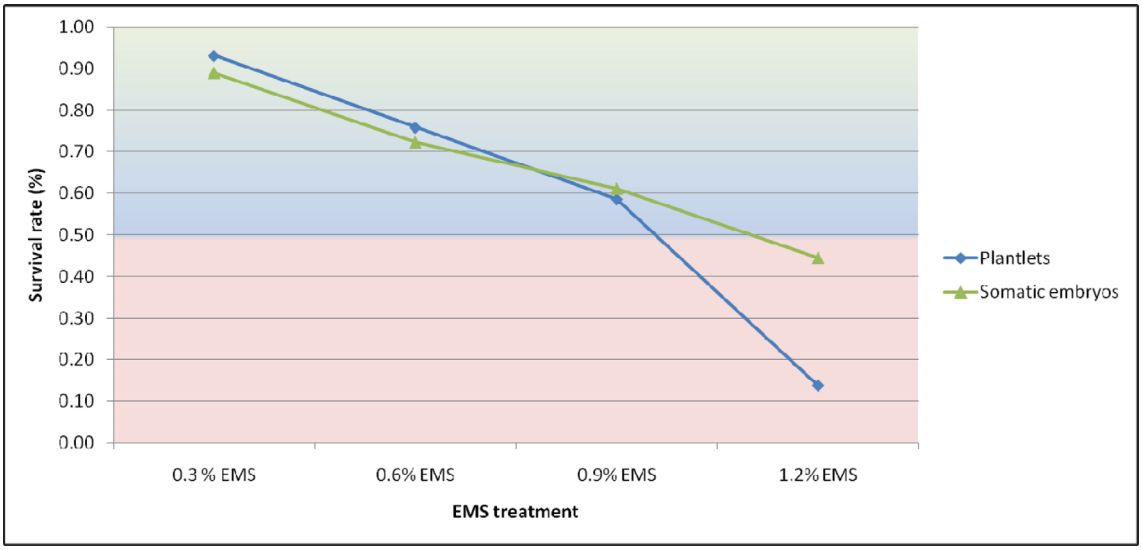

Figure 2. Response of cultures to EMS treatment in cassava

is suggested as a simple and preliminary option to monitor somaclonal variation in cassava. Subsequently green house and field evaluation are performed for either confirmation or identification of additional mutated traits (Reed et al., 2004).

The effect of *in vitro* mutagenesis in Sree Jaya through EMS treatment on somatic embryos (SE) after two weeks of treatment is shown in Table 1.The data clearly indicate that the higher concentration of 1.5 per cent EMS resulted in 100 per cent lethality in both the SE and SE derived plantlets. The observation taken at 7 weeks after treatment is presented in Table 2. Results indicated that there was no significant difference among the treatments with respect to number of roots produced per culture and the score for the size of the roots. There was reduction in plant height, number of shoots, leaves and length of roots in media incorporated with EMS. Thus, the effect of EMS as a mutagen is evident on

Table 1. Effect of Ethyl Methyl Sulpohonate on *in vitro* cultures in cassava at two weeks after treatment

Concentration of EMS (%)	Survival of mutagen treated cultures (%)		
	Somatic embyiod (SE)	Abnormal SE expressed as % of SE survived	SE derived plantlets
0.30% EMS	89.00	13.00	93.00
0.60% EMS	72.00	21.00	76.00
0.90% EMS	61.00	25.00	59.00
1.20% EMS	44.00	32.00	14.00
1.50% EMS	0.00	0.00	0.00

the tissue development, resulting in measurable physiological and morphological changes (Muthusamy et al., 2007) in *Arachis hypogaea* (groundnut).

Results of evaluation of plantlets derived from somatic embryos treated with EMS at various concentrations 7 weeks after treatment is presented in Table 3.The number of shoots and leaves per culture reduced when treated with EMS. Additionally, taller plantlets and longer roots were observed in media containing 0.3 per cent EMS. Lowest number of roots per culture (3.44) and shorter roots (2.33mm) were observed at 1.2 per cent concentration of EMS. Similar reduction on luck bomboo, *Dracaena sanderiana* (Junaid et al., 2008).

Results of effect of gamma irradiation on somatic embryo and somatic embryo derived plantlets of Sree Jaya at two weeks after treatment is shown in Table 4. The table shows that with increase of gamma radiation more than 60Gy resulted in complete mortality of the cultures. The effect of gamma irradiation on somatic embryos in Sree Jaya after seven weeks of culturing is presented in Table 5. Results indicated that the number of shoots and leaves per culture, number, length and size of the

Table 2. Evaluation of somatic embryos of cassava treated with Ethyl Methyl Sulpohonate at 7 weeks after treatment

Trait	Concentration of EMS (%)				
	Control	0.3	0.6	0.9	1.2
Height of the longest plantlet (cm)	3.36^{b}	1.18^{a}	1.02^{a}	0.94^{a}	0.36^{a}
No. of Shoots/culture	11.36^{b}	4.57^{a}	5.50^{a}	4.00^{a}	5.00^{a}
No. of leaves/culture	20.36^{b}	9.64^{a}	10.50^{a}	8.00^{a}	9.71^{a}
No. of roots/culture	2.93^{a}	0.50^{a}	0.71^{a}	0.86^{a}	1.86^{a}
Length of longest root (cm)	2.64^{b}	0.50^{a}	0.71^{a}	0.64^{a}	1.07^{a}
Size of roots (score 1-10)	1.14^{a}	0.50^{a}	0.71^{a}	0.57^{a}	0.71^{a}

Table 3. Evaluation of plantlets derived from somatic embryos of cassava treated with Ethyl Methyl Sulpohonate at 7 weeks after treatment

Trait	Concentration of EMS (%)				
	Control	0.3	0.6	0.9	1.2
Height of the longest plantlet (cm)	8.89^{c}	7.33^{bc}	5.89^{abc}	4.22^{ab}	2.67^{a}
No. of Shoots/culture	13.89^{b}	9.22^{a}	7.44^{a}	6.00^{a}	5.67^{a}
No. of leaves/culture	20.56^{b}	17.11^{b}	14.11^{a}	13.78^{a}	9.78^{a}
No. of roots/culture	7.56^{b}	5.89^{ab}	5.67^{ab}	4.56^{ab}	3.44^{a}
Length of longest root (cm)	5.22^{b}	5.00^{b}	4.78^{ab}	3.11^{ab}	2.33^{a}
Size of roots (score 1-10)	2.22^{a}	1.89^{a}	1.78^{a}	1.67^{a}	1.33^{a}

Table 4. Effect of gamma irradiation on *in vitro* cultures in cassava at two weeks after treatment

Dose of gamma treatment Gy	SE survived %	Abnormal SE as per cent of SE survived	Plantlets survived (%)
10	100.00	25.00	100.00
20	71.00	30.00	89.00
30	57.00	33.25	76.00
40	30.06	40.28	71.15
50	29.00	45.79	64.00
60	14.00	100.00	28.00
70	0.00	0.00	0.00
80	0.00	0.00	0.00
90	0.00	0.00	0.00
100	0.00	0.00	0.00
110	0.00	0.00	0.00

Gy – Gray

Table 5. Effect of Gamma irradiation at LD_{50} on somatic embryos in cassava at seven weeks after treatment.

	Dose of gamma ray irradiation		
Trait	Control	40Gy	50Gy
Height of the longest plantlet (cm)	1.44[b]	1.00[a]	1.00[a]
No. of Shoots/culture	11.11[a]	8.78[a]	3.78[a]
No. of leaves/culture	18.22[a]	8.33[a]	7.00[a]
No. of roots/culture	0.56[a]	0.00[a]	0.00[a]
Length of longest root (cm)	0.67[a]	0.00[a]	0.00[a]
Size of roots (score 1-10)	0.67[a]	0.00[a]	0.00[a]

Table 6. Effect of gamma irradiation at LD_{50} on plantlets derived from somatic embryo of cassava at 7 weeks after treatment

	Dose of gamma ray irradiation (Gy)		
Trait	Control	40Gy	50Gy
Height of the longest plantlet (cm)	5.44[b]	2.89[a]	1.94[a]
No. of Shoots/culture	24.00[b]	9.56[a]	5.67[a]
No. of leaves/culture	19.00[b]	17.78[b]	12.11[a]
No. of roots/culture	13.44[b]	7.22[a]	1.89[a]
Length of longest root (cm)	5.89[b]	2.67[a]	1.44[a]
Size of roots (score 1-10)	1.89[a]	1.11[a]	1.00[a]

roots did not differ with the treatments. Cultures which are not irradiated yielded plantlets with longer shoots. Irradiation of somatic embryos of the Sree Jaya with gamma rays caused a reduction of plantlet height.

The observations on the plantlets treated with gamma ray at LD_{50} value is presented in Table 6. The size of the roots did not differ between the treatments. Height of somatic embryo derived plantlets, number of shoots and leaves per culture, were higher in plantlets which were not irradiated. However, fewer and shorter roots were formed in cultures irradiated at 50Gy. Similar results in gamma irradiated and regenerated garlic cultures were observed (Zhen, 2001).

Evaluation of variability under ex vitro conditions

Mutants can be evaluated for survival rate, plant height, thickness of stem, number of nodes, number

Table 7. Evaluation of the *in vitro* mutants under pad and fan green house for quantitative traits at three months after planting

Genotype	Treatment	Height (cm)	Shoot no.	Leaf no.
S. Jaya	0.3% EMS	58	1	10
S. Jaya	0.3% EMS	45	1	8
S. Jaya	0.3% EMS	25	4	14
S. Jaya	0.3% EMS	33	1	10
S. Jaya	0.3% EMS	10	1	8
S. Jaya	1.2% EMS	30	1	10
S. Jaya	1.2% EMS	15	1	2
S. Jaya	1.2% EMS	50	1	5
S. Jaya	1.2% EMS	21	1	4
S. Jaya	1.2% EMS	21	1	3
S. Jaya	1.2% EMS	20	2	8

of leaves, leaf length, root number and or length and thickness of roots under green house conditions (Jorge et al., 2000 and Mapayi et al., 2013). Once the mutants are established in field conditions the IITA descriptors for cassava genotypes might be used for both characterization and performance evaluation against the original genotype. Since, each mutant plant has potential to become a new clone the data should be recorded at individual basis.

The *ex vitro* acclimatisation process was carried out under pad and fan green house, where the humidity and temperature conditions are kept constant throughout and not affected by the fluctuation of daily weather conditions. High humidity is maintained at above 85% and temperatures as low as about 27ºC. Although its maintenance costs are higher than conventional green house conditions, it has a fundamental advantage of granting higher survival rates for acclimatization of *in vitro* plantlets of cassava. Hardening of plantlets under tissue culture laboaratory resulted in no success while, under conventional green house three out of 79 plants were survived . However, under pad and fan green house 50 per cent of the plants established successfully.

Table 8. Evaluation of the cassava *in vitro* mutants under pad and fan green house for qualitative traits at three months after planting

Dose / concentration	Petiole colour	Stipule colour	Emerging leaf colour	Stem
Mother plant	Purple	Light green	Purplish green	Light brown
0.3% EMS	Light red	Light green	Green	Orange
0.3% EMS	Light red	Light green	Green	Orange
0.3% EMS	Yellowish green	Light green	Green	Green-yellowish
0.3% EMS	Yellowish green	Light green	Green	Green
0.3% EMS	Yellowish green	Light green	Green	Green
1.2% EMS	Pink	Light green	Light green	Light brown
1.2% EMS	Pink	Light green	Light green	Yellow
1.2% EMS	Red	Purplish green	Green	Brown
1.2% EMS	Pink	Purplish green	Purplish green	Light brown
1.2% EMS	Pink	Purplish green	Purplish green	Light brown
1.2% EMS	Pink	Purplish green	Purplish green	Light brown

Variation in the quantitative traits

The results on the evaluation of mutagen treated *in vitro* derived plantlets for quantitative traits at three months after planting (Table 7) showed that there are variations in the in plant height, number of branches and leaf number. In Sree Jaya, the mutated plants showed plant height varying between 10cm to 58cm. The shoot number varied between one and four and leaf number between two and fourteen. Similarly found variation in plant height and leaf area in mutated plants of cassava (Joseph et al., 2004).

Variation in the qualitative traits

The evaluation of qualitative traits at three months after planting showed that there are variations in colour of petiole stipule, emerging leaf and stem as presented in Table 8. In Sree Jaya, the wild type was having petiole colour purple while in the mutated plants there were light red, yellowish green, pink and red coloured petiole. Stipule colour changed from light green of the wild type to purplish green in few mutated plants while others had light green stipule colour. Emerging leaf colour in the

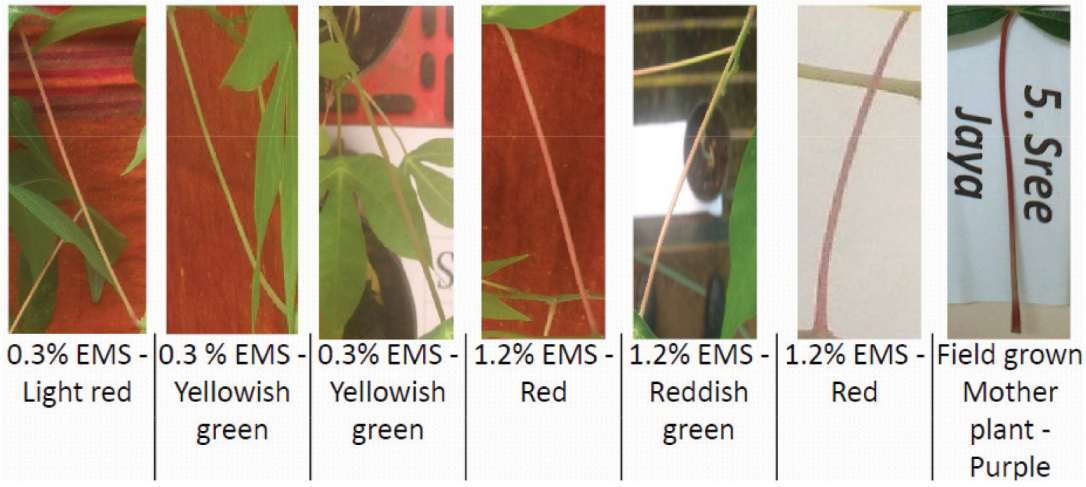

Plate 1. Variation in petiole colour of *in vitro* mutants of Sree Jaya at three months after planting out

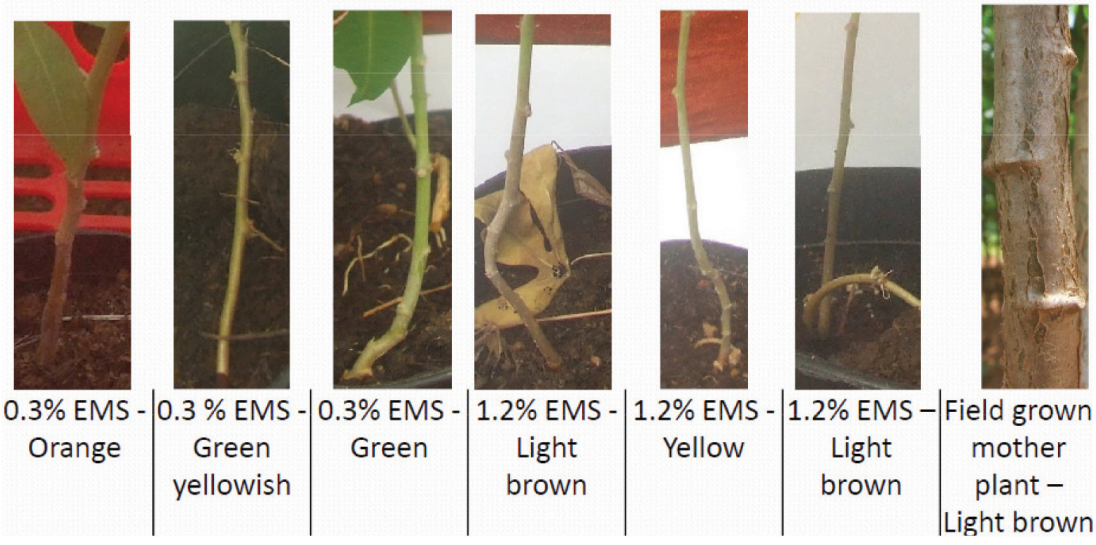

Plate 2. Variation in stem colour of *in vitro* mutants of Sree Jaya at three months after planting out

wild type was purplish green, while, the mutated plants showed variations as green and light green. In wild type stem colour was light brown, while the mutated plants showed variations as orange, greenish yellow, green, light brown, yellow and green (Plates 1 and 2). Similar results were obtained where the emerging leaf lobe colour varied from purple-green to light green, the petiole colour from purple-green to light green (Joseph et al., 2004).

As per the present study, in cassava *in vitro* mutagenesis, the LD_{50} value for gamma radiation was 30 Gy and 50 Gy for somatic embryoids and plantlets, respectively. LD_{50} value for EMS was 1.20 per cent for somatic embryoids and 0.90 per cent for plantlets. Variability with respect to quantitative traits like height, number of shoots and number of leaves were observed for *in vitro* derived plantlets in the hardening stage under pad and fan green house structure. The qualitative traits like colour of the petiole, stipule, emerging leaf and of the stem also varied between the mutated plants.

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