

# Effect of Alkylating Mutagens on Rooting Response and Callus Age on Shoot Regeneration of Rough Lemon (*Citrus Jambhiri* Lush.)

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## Authors' contributions

This work was carried out in collaboration among all authors. Author MI prepared the main parts of the manuscript being 'effect of alkylating mutagens on rooting response and callus age on shoot regeneration of rough lemon (*Citrus jambhiri* Lush.)' of his PhD project. Author PB was supervisor of the student provided materials and methods of the study and statistical analysis. Authors KK and BKS contributed to manuscript elaboration for the transformation and regeneration paragraph. Author MI coordinated and wrote the manuscript. All authors read and approved the final manuscript.

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## ABSTRACT

Many species of *Citrus* and compatible sexual relatives are being used to develop biotic and abiotic tolerant rootstocks and their ability to confer positive stionic effects. *Citrus jambhiri* is the commercial citrus rootstock in India, deep-rooted well adapted to the diverse agro-climatic conditions. It ensures high yield with large size fruits in most of the scion cultivars and at the same time is resistant to most of the viruses. For *in vitro* mutagenesis, leaf and epicotyl calli derived shoots were used as explant material. *In-vitro* mutagenesis is a valuable tool for improvement of a crop, especially when there is a need to add one or two easily identifiable characters in an

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otherwise well adapted variety, without disturbing its basic genotype. The alkylating agent methyl methane sulphonate (MMS) and ethyl methane sulphonate (EMS) at 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6%, were used for mutagenesis. The mutagenic calli derived shoots were regenerated on MS medium augmented with BAP (3.0 mg/l), followed by rooting in MS medium containing NAA (2.0 mg/l). Percent rooting (29.50-8.33%), (27.11-07.72%), number of roots per shoot (3.11-1.18), (3.12-1.04) and root length (4.13-2.22), (4.15-2.17) decreased with increasing doses of MMS and EMS treatments, respectively. Effect of increasing age of callus showed that callus retained regeneration capacity (3.55%) even after 210 days of culture by repeated sub-culturing. The plantlets were successfully acclimatized in different potting mixtures and highest survival rate (90.35%) was achieved in potting mixture containing garden soil with sand and farmyard manure (1:1:1).

**Keywords:** Rough lemon; callus; regeneration; mutagens; root; tissue culture.

## 1. INTRODUCTION

Rough lemon (*Citrus jambhiri*) is the most commonly used rootstock for various scion cultivars of citrus in all over the world. It is native to North-eastern India, probably a natural hybrid because of its high degree of polyembryony compared to other lemon species. Rough lemon rootstock is suited to deep well drained sandy soil which are subject by the marked fluctuations in soil moisture [1]. It forms a normal union with all scion varieties, develops a deep root system, produces heavy yields and gives a long life to trees when planted on a suitable soil. It imparts resistance to tristeza and exocortis, viroid, tolerant to salt and drought. It ensures high yield with large size fruits in most of the scion cultivars and at the same time is resistant to most of the viruses [2]. It is an important rootstock for a number of citrus fruit crops including oranges, grape fruits, kinnows, lemons, and mandarins all over the world [1] and is highly vulnerable to *Phytophthora*, which leads to main losses in an orchard if appropriate phyto-sanitary conditions are not followed [3-5]. The potential of conventional methods of upgrading of citrus rootstocks is limited by biological factors that hinder breeding and selection, such as heterozygosity and inbreeding pollen and ovule sterility, sexual incompatibility, apomixis, depression, nucellar polyembryony and juvenility [4].

*In-vitro* mutagenesis is a valuable tool for improvement of a crop, especially when there is a need to add one or two easily identifiable characters in an otherwise well adapted variety, without disturbing its basic genotype [6]. In fruit crops, mutagenesis has already been used to induce many useful traits affecting plant size, blooming time, fruit colour, fruit ripening, self-incompatibility and resistance to pathogens [7]. Ethyl methane sulfonate (EMS) and Methyl

methane sulfonate (MMS) enhances genetic variability which can enhance resistance against pathogens in crop plants [6]. A lot of work has been done to induce mutations artificially by EMS and MMS in crop plants. Seed mutagenesis has been used for induction of early flowering in spring rape, herbicide tolerance in soybean, male sterility in wheat and cucumber, increased pollen variability and fruit rot resistance in bell pepper as well as quantitative variations in different yield traits in *Avena sativa* L [7].

Considering the susceptibility of *Citrus jambhiri* to *Phytophthora* spp. and the potential of *in-vitro* mutagenesis to produce stable genetic variants, the present investigation was carried out with the objective of induction of variations in the *in vitro* cultures of *Citrus jambhiri* by employing chemical mutagens and regeneration status of callus.

## 2. MATERIALS AND METHODS

The present investigation was carried out at Plant Tissue Culture Laboratory, Division of Fruit Science and School of Biotechnology in collaboration with Division of Plant Pathology, Sher-e-Kashmir University of Agricultural Sciences and Technology of Jammu during 2016 to 2018.

### 2.1 Planting Materials

The leaf and epicotyl segments were treated with Tween 20 (2-3 drops/100 ml of water) for five minutes and washed thoroughly with distilled water to remove surface contaminants. After that, the explant segments were treated with (0.2%) carbendazim for five minutes and washed thoroughly in running tap water for ten minutes. Finally, sterilization procedures were carried under aseptic conditions in a laminar air flow chamber and explants were treated with mercuric chloride 0.1% for three minutes followed by

washing with distilled water and subsequent dipping in Ethanol 70% for thirty seconds. Callus was initiated in 25x150 mm culture tubes containing 40 ml of MS medium supplemented with 2,4-D (3.0 mg/l). After inoculation, the vessels were kept in culture room at  $25\pm 2^{\circ}\text{C}$  under 16 hours photoperiod and 1.5-2.0 kilo light intensity.

Leaf and epicotyl derived callus was regenerated and used for mutagenesis (Fig. 1, 2 and 3). Six doses (0.1%, 0.2%, 0.3%, 0.4%, 0.5% and 0.6%) each of the two chemical mutagens, ethyl methane sulphonate (EMS) and methyl methane sulphonate (MMS) were used to study the effect of chemical mutagens on rooting of shoots derived from mutagenic callus.

## 2.2 EMS and MMS Treatment

For 0.1 percent EMS/MMS treatment, 1 ml of filter sterilized EMS/MMS was added to one litre of autoclave liquid (0.22-micron Millipore) sterilized using filtered assembly connected to suction pump. Mutagens were thoroughly mixed with medium by gentle shaking; it was then poured into autoclaved culture tubes. Calli of uniform size were dipped in EMS/MMS for six hours and jars were put on shakers. Likewise, other treatment doses of EMS/MMS were given to the calli. After the treatment for specified duration, the mutagenised calli were cultured on MS medium supplemented with 2,4-D (3.0 mg/l). Then the calli were transferred to the regeneration medium LD<sub>50</sub> doses were optimized by taking into account the mortality of the calli.

## 2.3 Rooting of Regenerated Shoots

For rooting, the regenerated shoots were separated out and cultured on MS medium (half strength) supplemented with NAA (2.0 mg/l).

## 2.4 Hardening and Acclimatization

Regenerated plantlets were washed with double sterile distilled water in order to remove any adhering medium and then transferred to autoclaved plastic pots containing autoclaved coco peat, vermiculite and perlite in the ratio of 1:1:1. Hardening of potted plantlets was accomplished in culture room set at  $25\pm 2^{\circ}\text{C}$  and 16-hours day length by covering them with polyethylene bags to maintain high humidity. After 12-15 days, polyethylene bags were removed initially for a short duration (15-30 min) daily for about one week. Gradually, the daily exposure time was increased by 30 min for each day. Polyethylene bags were completely removed after 20 days. Subsequently, the plantlets were transferred to earthen pots containing only garden soil and kept in the greenhouse for one month for acclimatization, and then transferred to screen house.

## 2.5 Statistical Analysis

The data were analysed according to Completely Randomized Block design (CRD) [8]. Data scored on percentage wherever necessary were subjected to the arc sine transformation for the analysis of variance (ANOVA).

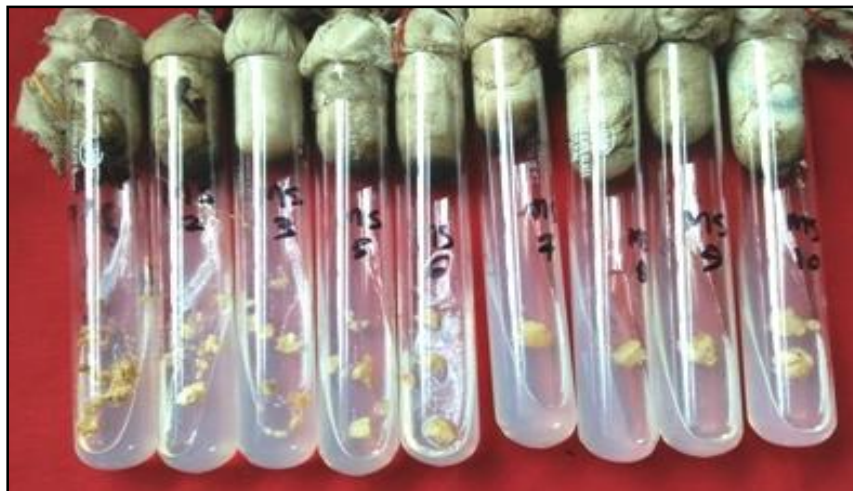


Fig. 1. Survival of calli on MS medium with 0.5 % MMS



Fig. 2. Survival of calli on MS medium with 0.5 % EMS



Fig. 3. Regeneration of mutagenic callus on MS medium supplemented with BAP (3.0 mg/l)

### 3. RESULTS

#### 3.1 Effect of MMS on Rooting of Shoots Regenerated from Mutant Calli of Leaf and Epicotyl Segments

The data regarding effect of methyl methane sulphonate (MMS) treatment on rooting of shoots regenerated mutant calli of leaf and epicotyl segments of *Citrus jambhiri* on MS (half strength) medium is presented in Table 1 and depicted in Fig 4. It is obvious from the data that among seven treatments, maximum rooting response (94.63%) was recorded on MS (half strength) medium supplemented with NAA (2.0 mg/l) without mutagen and was significantly higher as compared to all other treatments, followed by

rooting percentage of 29.50 in 0.1% dose of MMS treatment and no rooting response was recorded beyond the 0.5% dose of MMS treatments. Maximum numbers of roots per shoot (3.85%) were obtained from control (MS half strength medium supplemented with NAA 2 mg/l without mutagen) and was at par with number of roots per shoot of 3.11 in 0.1% dose of MMS treatment, whereas no roots per shoot were observed beyond the 0.5% dose of MMS treatments. Regarding root length, maximum root length (4.55 cm) was recorded on MS (half strength) medium supplemented with NAA (2 mg/l) without mutagen and was at par with root length of 4.13 cm in the 0.1% dose of MMS treatment.



### 3.2 Effect of EMS on Rooting of Shoots Regenerated Mutant Calli of Leaf and Epicotyl Segments

The data pertaining to the effect of ethyl methane sulphonate (EMS) treatments on rooting of the shoots regenerated from mutant calli of leaf and epicotyl segments of *Citrus jambhiri* on MS (half strength) medium is presented in Table 2 and Fig. 5. The perusal of the data revealed EMS treatments has significant effect on rooting response, number of roots per shoot and root length. Among seven treatments, maximum rooting response of 94.77% was recorded on MS (half strength) medium supplemented with NAA (2.0 mg/l) without mutagen and was significantly higher as compared to all other treatments, followed by rooting percentage of 27.11 in 0.1%

EMS treatment and no rooting response was recorded beyond the 0.5% dose of EMS treatment. Numbers of roots per shoot were decreased significantly with the increase in the dose of EMS treatments. Maximum numbers of roots per shoot (3.79%) were obtained from control (MS half strength medium supplemented with NAA 2.0 mg/l without mutagen) and was at par with number of roots per shoot of 3.12 in 0.1% dose of EMS treatment, whereas no roots per shoot were observed beyond the 0.5% dose of EMS treatments. Regarding root length, maximum root length (4.51 cm) was recorded on MS (half strength) medium supplemented with NAA (2 mg/l) without mutagen and was at par with root length of 4.15 cm in the 0.1% dose of EMS treatment.

**Table 1. Effect of methyl methane sulphonate (MMS) treatment on rooting from the shoots regenerated from mutagenic callus of *Citrus jambhiri* Lush. on half strength MS medium**

MMS (%)	Rooting <sup>a</sup> (%)	No. of roots/shoot	Root length (cm)
0.1%	29.50 (33.05)*	3.11	4.13
0.2%	24.17 (29.38)	2.36	3.27
0.3%	21.84 (27.840)	2.01	2.91
0.4%	16.67 (23.98)	1.82	2.73
0.5%	8.33 (16.44)	1.18	2.22
0.6%	00.00 (00.00)	-	-
Control (MSA)**	94.63 (77.32)	3.85	4.55
CD <sub>(0.05)</sub>	3.11	1.37	1.24

\* Values in parenthesis are Arc sine transformed values; \*\* MSA-half strength Murashige and Skoog medium supplemented with NAA (2 mg/l) without mutagen; <sup>a</sup> Rooting on Murashige and Skoog medium supplemented with NAA (2 mg/l)



**Fig 4. Rooting response of shoots regenerated from mutagenic callus on MS medium supplemented without mutagen**

**Table 2. Effect of ethyl methane sulphonate (EMS) treatment on rooting from the shoots regenerated from mutagenic callus of *Citrus jambhiri* Lush. on half strength MS medium**

EMS (%)	Rooting <sup>a</sup> (%)	No. of roots/shoot	Root length (cm)
0.1%	27.11(31.32)*	3.12	4.15
0.2%	21.43 (27.46)	2.21	3.32
0.3%	19.66 (26.24)	1.96	2.84
0.4%	16.19 (23.61)	1.55	2.66
0.5%	07.72 (15.75)	1.04	2.17
0.6%	00.00 (00.00)	-	-
Control (MSA) **	94.77 (77.54)	3.79	4.51
CD <sub>(0.05)</sub>	3.21	1.51	1.18

\* Values in parenthesis are Arc sine transformed values; \*\* MSA-half strength Murashige and Skoog medium supplemented with NAA (2 mg/l) without mutagen; <sup>a</sup> Rooting on Murashige and Skoog medium supplemented with NAA (2 mg/l)



**Fig. 5. Rooting response of shoots regenerated from mutagenic callus on MS medium supplemented without mutagen**

**Table 3. Effect of callus age on shoot regeneration in *Citrus jambhiri* Lush. on MS medium**

Callus age (days)	Shoot regeneration <sup>a</sup> (%)	No. of shoots/culture	Days to regenerate shoots
60	42.33 (40.55) *	3.28	40.22
90	34.14 (35.72)	3.02	57.83
120	20.08 (26.54)	2.71	89.08
150	15.01 (22.59)	2.19	122.04
180	9.63 (17.82)	1.99	158.77
210	3.55 (10.54)	0.55	180.11
CD <sub>(0.05)</sub>	6.07	0.25	12.46

\* Values in parenthesis are Arc sine transformed values; <sup>a</sup> Regenerated on Murashige and Skoog medium supplemented with BAP (3.0 mg/l)

### 3.3 Effect of Callus Age on Shoot Regeneration

To study the effect of callus age on shoot regeneration capacity in *Citrus jambhiri*, callus of

different age groups were regenerated on regeneration medium of MS medium supplemented with BAP (3.0 mg/l). The results of the percent shoot regeneration, number of shoots per culture and days to regenerate shoots

are presented in Table 3 and Fig. 7. It is evident from the data that percent shoot regeneration and number of shoots per culture decreased with increase in the age of callus. The maximum shoot regeneration (42.33%) was recorded in 60

days old callus on regeneration medium, followed by shoot regeneration percentage of 34.14 in 90 days old callus. The minimum shoot regeneration of 03.55% was recorded in 210 days old callus on regeneration medium.

**Table 4. Influence of different potting mixtures on survival of the plantlets of *Citrus jambhiri* Lush**

Treatment	Survival percentage
Garden Soil	38.92 (38.58) *
Garden Soil + Sand (1:1)	72.27 (58.32)
Garden Soil + Sand (1:2)	70.11 (56.89)
Garden Soil + Sand (2:1)	64.47 (53.41)
Garden Soil + FYM (1:1)	77.09 (61.47)
Garden Soil + FYM (1:2)	68.28 (55.72)
Garden Soil + FYM (2:1)	70.26 (56.98)
Garden Soil + Sand + FYM (1:1:1)	90.35 (72.28)
Garden Soil + Sand + FYM (1:2:1)	79.64 (63.22)
Garden Soil + Sand + FYM (2:1:1)	86.22 (68.51)
Garden Soil + Sand + FYM (1:1:2)	72.58 (58.52)
CD <sub>(0.05)</sub>	3.37

\* Values in parenthesis are Arc sine transformed values; FYM: Farm Yard Manure



**Fig. 6. Soma clonal plants transplanting in plastic pots**



**Fig. 7. 210 days old callus and its regeneration of 210 days old callus**

### 3.4 Influence of Potting Mixtures on Survival of the Plantlets

The data pertaining to Influence of different potting mixtures on survival of the plantlets of *Citrus jambhiri* is presented in Table 4 and depicted in Fig. 6. The perusal of the data revealed that different kinds of potting mixture were found to influence significantly on survival percentage of rough lemon plantlets. Among different treatments of potting mixtures, maximum (90.35%) survival of plantlets were recorded in the potting mixture containing garden soil, sand and FYM (1:1:1), followed by plantlet survival percentage of 86.22 in the potting mixture of garden soil, sand and FYM (2:1:1). The potting mixture containing garden soil alone was significantly inferior to other potting mixtures with the plantlet survival of 38.92%.

## 4. DISCUSSION

*In-vitro* mutagenesis is considered as a valid tool for the improvement of a crop to add one or more easily identifiable characters without changing the genotype of well-developed variety. In addition, there is no loss of the mutants, as micro propagules are sub-cultured under sterile conditions [6]. Mutation breeding research is more effective than hybridization even when desired genes are present, but tightly to undesirable genes. The frequency of occurrence of mutation by the use of mutagen may as higher as 300 times than the occurrence of natural frequency seen [7]. Hence, attempts have been made to accelerate the rate artificially by means of physical and chemical mutagens. The frequency and saturation of mutations can be regulated by varying the mutagen dose [9] and

mutagenic agents can encourage different extensions of genomic lesions, ranging from base mutation to bigger fragments insertions or deletions [10]. Our findings are in line with the findings of Daniela [11] wherein the chemical mutagens showed better response for *in vitro* regeneration and development of variability in crop plants. Similarly, Xavier et al. [12] showed EMS to be quite capable as regards high mutation rate, especially related to chlorophyll changes during the *in vitro* condition. Humera and Iqbal [13] reported similar observations on *in vitro* mutation induction rate from chemical mutagens, regarding variations in height of shoot and number of shoots/roots in germinated seed.

The results obtained in the present investigation are similar to those of Savita et al. [14] in rough lemon. They reported 0.1% each (EMS and MMS) as most appropriate dose for 45 days old calli based on the survival and the regeneration potential. Similarly, Khokhar [15] reported that with increase in the dose of EMS, number of leaves and branches were decreased. Variations for different morphological characters were probably due to phenotypically constructive multidirectional mutations of polygenes caused by mutagen EMS and MMS. The varying response of plants after EMS and MMS treatments may be attributed to the differential sensitivity of different loci among the genotype for same or different characters. With increasing dose of EMS and MMS treatment, the percent germination, plant height, internodal length, number of leaves and primary root length were decreased as reported in *Citrus jambhiri* Lush [16]. These results are also in consonance with the findings of Kaur [17] wherein, percent regeneration, number of buds, number of shoots,



shoot length, number of leaves, internodal length, primary root length and number of secondary roots decreased with increasing dose of chemical mutagens (MMS and EMS) in epicotyl segments of rough lemon.

Regarding effect of callus age on shoot regeneration our results are in conformity with those of Savita et al. [18] wherein, the regeneration capacity of the callus decreased with increasing age of the callus. They also reported that the callus induced from cotyledons of *C. jambhiri* could be maintained in culture for more than a year and was found to regenerate (in 58 % of cultures) even after 420 days in culture. Similarly, Kumar et al. [14] reported significant decline in shoot regeneration and average number of regenerated shoots per callus with the increase in callus age in rough lemon.

Normah et al. [19] reported 83.33 % survival of regenerated plantlets of *Citrus halimii* under *ex-vitro* conditions. Al-Khayari and Al-Baharany [20] reported 90 % survival of regenerated plantlets of *Citrus aurantifolia*. Filho et al. [21] reported 100% survival in Sweet Orange. Rani et al. [22] reported 67% survival rate of rooted plantlets of Kinnow. Altaf et al. [2] reported 76 % survival of regenerated plantlets of *Citrus jambhiri*.

## 5. CONCLUSION

From the present investigation it can be concluded that the dose required for selection of tolerant calli corresponded upto 0.5 % each for MMS and EMS treatments and under *in vitro* conditions, an average of about 90.35 % of selected mutant regenerates exhibited soma clonal variation. It can also be concluded that leaf and epicotyl derived callus can regenerate even after 210 days of culture. The present investigation also reveals that alkylating mutagens can be potentially employed as a potent selection agent for carrying out *in vitro* selection system which can possibly be an alternative solution to problems like susceptibility of rough lemon to *Phytophthora* spp.

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## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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