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Validation of nucellus-based direct somatic embryogenesis mutagenesis and phenotypic assessment of M1 mutants in kinnow mandarin (*Citrus nobilis* × *Citrus deliciosa*)

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ABSTRACT

In ovulo nucellus based direct somatic embryogenesis (DSE) is an efficient tool to assess mutagenic efficiency in perennial crops. A study was carried out in 2022–23 and 2023–24 at ICAR-Indian Agricultural Research Institute, New Delhi to compare the influence of 0.1% ethyl methane sulphonate (EMS) for 5 h and gamma irradiation (80 Gy) on embryogenesis, plantlet regeneration, and growth traits in kinnow mandarin (*Citrus nobilis* × *Citrus deliciosa*). Both treatments significantly reduced ovule survival compared with control (86.67%). The survival declined to 48.00% under EMS (0.1% for 5 h) and 35.78% following gamma irradiation (80 Gy for 10.9 min). The frequency of direct somatic embryogenesis declined to 49.07% under EMS and 43.85% under gamma irradiation. In contrast, post embryogenic parameters, including germination and bipolar conversion efficiency, were more severely affected, decreasing to 39.06–41.89% under EMS and 32.87–30.14%, under gamma treatment. Regenerants displayed short stature, reduced internodal length, and smaller leaves, though leaf number remained unaffected, suggesting that stress mainly altered expansion and elongation rather than leaf initiation. Correlation analysis indicated that biomass under EMS was closely linked to leaf area, while gamma treatment weakened relationships between height related traits and biomass. Cluster analysis revealed moderate variability under EMS but greater divergence among gamma irradiated populations. Overall, EMS generated point mutations with moderate phenotypic effects, whereas gamma rays exerted stronger disruptive impacts on regeneration and variability. These findings demonstrate that *in ovulo* nucellus-derived DSE is a robust system for evaluating mutagenic efficiency and optimizing treatments for generating targeted variability in perennial fruit crops.

Keywords: Cluster analysis, Correlation, Direct somatic embryogenesis, EMS, Gamma, *In ovulo* nucellus, Regenerants

Kinnow (*Citrus nobilis* Lour × *Citrus deliciosa* Tenora), a mandarin hybrid bred in California, is well known for its high yield potential, wider adaptability, higher juice recovery, nutritional quality, and export potential (Singh *et al.* 2023). This 'golden fruit' has revolutionised the citrus industry of Punjab and accounts for over 93% total citrus acreage in the state, with south-western arid regions being the leading production areas (Kumar *et al.* 2025). The seed-laden nature of kinnow (30–35 seeds/fruit), however, makes them less demanding in processing sector, which necessitates the development of low seeded varieties (<10 seeds/fruit) (Kumar *et al.* 2021).

Mutagenesis as a tool is an effective breeding strategy for trait-oriented advancement in citrus (Roose and Williams 2007). A significant advancement in seedlessness has been reported in 'Kozan' common orange through induced physical mutation, with the trait stabilised by the third generation (Cimen *et al.* 2021). Conventional *ex vitro* mutagenesis is often constrained by chimerism and low efficiency, whereas *in vitro* techniques enable the precise treatment of isolated cells, thereby enhancing the recovery of stable mutants (Penna *et al.* 2012). Somatic embryogenesis is an asexual developmental process in which embryos arise from somatic/vegetative cells without fertilization, either directly from tissues or indirectly via callus, and serve as a powerful tool for regeneration (Ikeuchi *et al.* 2019). Indirect somatic embryogenesis (ISE) via callus is prone to somaclonal variations, leading to off-types and loss of genetic fidelity, thereby limiting its use for clonal propagation (Piccioni *et al.* 1997). However, in direct somatic embryogenesis (DSE), embryo develops directly from explant cells, bypassing callus formation, through a pro-embryogenic mass (PM) capable of high proliferation (Ferrari *et al.* 2021). A robust

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in ovulo nucellus based specialised DSE system has been developed by Murugan *et al.* (2023), allowing efficient single cell regeneration through retrieval of micropylar region-zygotic embryos at stage III [>21–25 mm, 50–70 days after anthesis (DAA)]. Morphological observations confirmed the recovery of homohistont and molecular analysis using ISSR markers identified its genetic uniformity, indicating the reliability of the developed regeneration protocol. Non-chimeric regeneration through *in vitro* mutagenesis was successfully established in kinnow, and the optimum mutagen concentration for EMS and Gamma was determined to be 0.1% for 5 h and 80 Gy for 20 min, respectively (Theivani 2023, Murugan *et al.* 2024). In this backdrop, the present investigation aimed to validate and develop a broad M1 population for recovering stable non-chimeric mutants, and to screen them morphologically prior to molecular validation.

MATERIALS AND METHODS

Explant preparation: Based on the standardised protocol, *in ovulo* nucellus was obtained by excising embryos from stage III kinnow fruits (21–25 mm) from an 8-year-old mother plant budded on rough lemon rootstock during May-June 2022–23 and 2023–24.

EMS and gamma treatment of explant: An aggregate of 450 ovules were treated with ethyl methane sulphonate (EMS) 0.1% for 1, 3 and 5 h at the Central Tissue Culture Laboratory, ICAR-National Institute for Plant Biotechnology, New Delhi. For each treatment (~100 ovules/concentration), ovules were placed in 150 mL conical flasks containing twice the volume of sterile EMS solution (*p*H 6.8–7.0), in triplicate. The flasks were cotton plugged, sealed with parafilm®, and agitated at 120 rpm on an orbital shaker to ensure uniform exposure. Ovules were aseptically collected at an interval of 1, 3 and 5 h, and washed five times with sterile distilled water (5–10 min each) to eliminate residual EMS. Similarly, a total of 450 ovules [5 ovules/plate, 3 replications, 15 plates/treatment] were inoculated on induction-cum-maturation medium (ICM) and irradiated with gamma rays at the Nuclear Research Laboratory, Division of Environment Science, ICAR-Indian Agricultural Research Institute (28°63' N, 77°08' E; at an elevation of 228.61 m amsl), New Delhi. Irradiation was carried out using a gamma chamber (GC-5000, Co^{60} source, $T_{1/2}$- 5.3 years) at a dose rate of 0.441 kGy/h (Chamber Temperature: 34.5°C), corresponding to an exposure of 80 Gy for 10.9 min.

Downstream handling: Following washing, 15 *in ovulo* nucellus explants per EMS and gamma treatment were cultured on somatic embryogenesis medium and incubated in darkness at 25 ± 2°C with 75–85% relative humidity.

Media constitution for mutant regeneration: Mutants were regenerated following Murugan *et al.* (2023), and the embryogenic response to physical and chemical mutagens was assessed on ICM (DKW + 5.0 mg/L kinetin + 1000 mg/L malt extract). After maturation, 25 uniform embryos/replicate were transferred to germination conversion medium (MT + 2.0 mg/L GA_3 + 0.5 mg/L NAA + 100 mg/L spermidine + 10% CW). Bipolar seedlings were shifted from dark to light, placed on filter paper bridges in basal liquid establishment medium, and subsequently hardened in 250 ml bottles containing cocopeat: vermiculite: perlite (2:1:1). Sequential observations on EMS and gamma influence were documented throughout the regeneration process in triplicate and were expressed as efficiency (%).

$$\text{Embryogenesis efficiency (\%)} = \frac{\text{No. of embryo initiating explants}}{\text{No. of post irradiation survivors}} \times 100$$

$$\text{Germination efficiency (\%)} = \frac{\text{No. of primordia initiating embryos}}{\text{No. of total embryos placed on germination medium}} \times 100$$

$$\text{Bipolar conversion efficiency (\%)} = \frac{\text{No. of embryos developing shoots and roots >3 cm}}{\text{No. of embryos germinated}} \times 100$$

$$\text{Plantlet establishment efficiency (\%)} = \frac{\text{No. of whole/complete emblings obtained}}{\text{No. of bipolar seedlings transferred to establishment medium}} \times 100$$

$$\text{Acclimatization efficiency (\%)} = \frac{\text{No. of survived emblings}}{\text{No. of establishment emblings transferred to hardening medium}} \times 100$$

Phenotypic assessment of M1 population: Primary hardened plants were pooled from 2022–23 and 2023–24 and their morphological traits were evaluated. Plant height and internodal length were recorded using a metre scale, while leaf length, width and area were assessed with WINFOLIA Pro 2020a (leaf image analysis software). Fresh weight was recorded using electronic balance.

Statistical analysis: The experimental data for two consecutive years were subjected to a pooled analysis of variance (ANOVA) under a completely randomised design (CRD) using Indostat software, following a Bartlett's test to confirm the homogeneity of variances. Mean separations were performed using Tukey's Honestly Significant Difference (HSD) test at a significance level of ≤ 0.05 to identify significant differences between genotypes and years. To assess genetic diversity among the 54 EMS-induced and 56 gamma-irradiated genotypes, Hierarchical Cluster Analysis (HCA) was executed using Ward's minimum variance method through IASRI-Statistical Package for Agricultural Research (SPAR 2.O) software. Within the same package, a heatmap was generated to visualise the inter-relationships and correlation patterns among the evaluated morphological parameters of the regenerants. Furthermore, a Principal Component Analysis (PCA) was conducted on the pooled data, and a combined biplot for both the years was generated using Agri-Analyse platform to illustrate the spatial distribution of genotypes and the contribution of specific traits to total observed variation.

RESULTS AND DISCUSSION

Comparative mutagenic efficiency in in ovulo *nucellus direct somatic embryogenesis*: Both EMS and

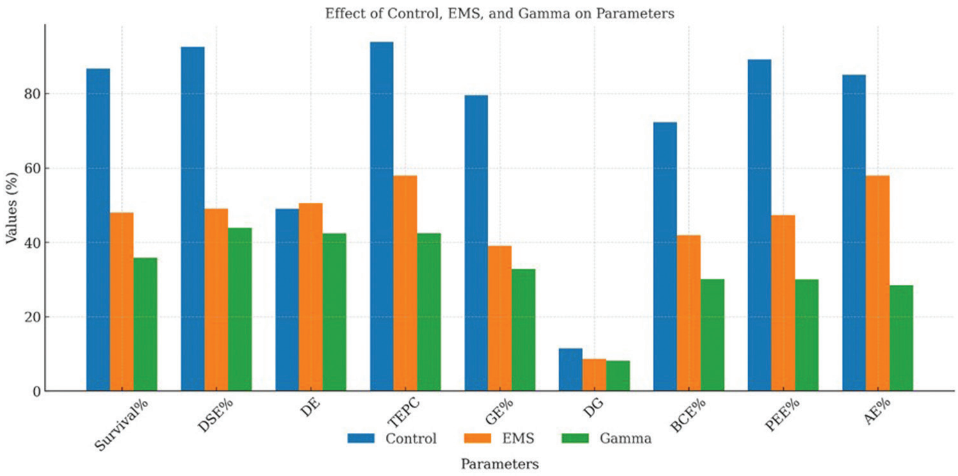

Fig. 1 Responses of *in vitro* direct somatic embryogenesis to EMS and gamma irradiation treatments based on pooled data from two consecutive years.
DSE, Direct somatic embryogenesis; DE, Days to embryogenesis; TEPC, Total embryo production capacity; GE, Germination efficiency; DG, Days to germination; BCE, Bipolar conversion efficiency; PEE, Plantlet establishment efficiency; AE, Acclimatisation efficiency.

gamma treatments significantly reduced ovule survival, direct somatic embryogenesis, and embryo establishment efficiency (Fig. 1). Ovule survival decreased from 86.67% in the control to 48.0% under 0.1% EMS and to 35.78% under 80 Gy gamma irradiation. The greater reduction under gamma irradiation may be because EMS, a chemical alkylating agent, primarily induces point mutations (G→A transitions) whereas gamma rays, as ionizing radiations, generate free radicals and cause extensive chromosomal aberrations (Neeraj *et al.* 2021). Supporting this observation, a study in black gram demonstrated that EMS possessed 2–2.5 fold higher efficiency than gamma rays based on ovule and pollen sterility assessments (Gautam and Sood 1992).

Direct somatic embryogenesis (DSE%), total embryo production capacity (TEPC), germination (GE%), and bipolar conversion efficiency (BCE%) showed marked reductions in response to mutagenic treatments. Across multiple species, gamma irradiation has been shown to reduce the average number of somatic embryos, as reported in avocado, citrus and coffee (Witjaksono and Litz 2004, Agisimanto *et al.* 2016, Nkurunziza *et al.* 2025). Furthermore, morphological conversion and establishment parameters [BCE%, Plantlet establishment efficiency (PEE%), Acclimatisation efficiency (AE%)] also declined significantly with mutagen exposure. While 0.1% EMS reduced these indices to 41.89–57.94%, 80 Gy gamma irradiation further lowered them to 30.14–28.5%, reflecting the stronger disruptive effect of ionizing radiations on post-embryogenic conversion and plantlet establishment.

PCA biplot: The combined Principal component analysis (Fig. 2) demonstrated that the multivariate response of *in ovulo* nucellus-derived somatic embryogenesis is governed primarily by regeneration efficiency traits with PC1 (47.59%) separating treatments with high embryogenic competence and successful plantlet regeneration (DSE%, Germination efficiency, BCE, PEE and AE%) from those characterized by delayed development and reduced survival (Days to embryogenesis and Survival%). PC2 (14.34%) captured secondary variation associated mainly with developmental timing and stress response. Trait relationships remained consistent across both years, as indicated by similar loading directions. However

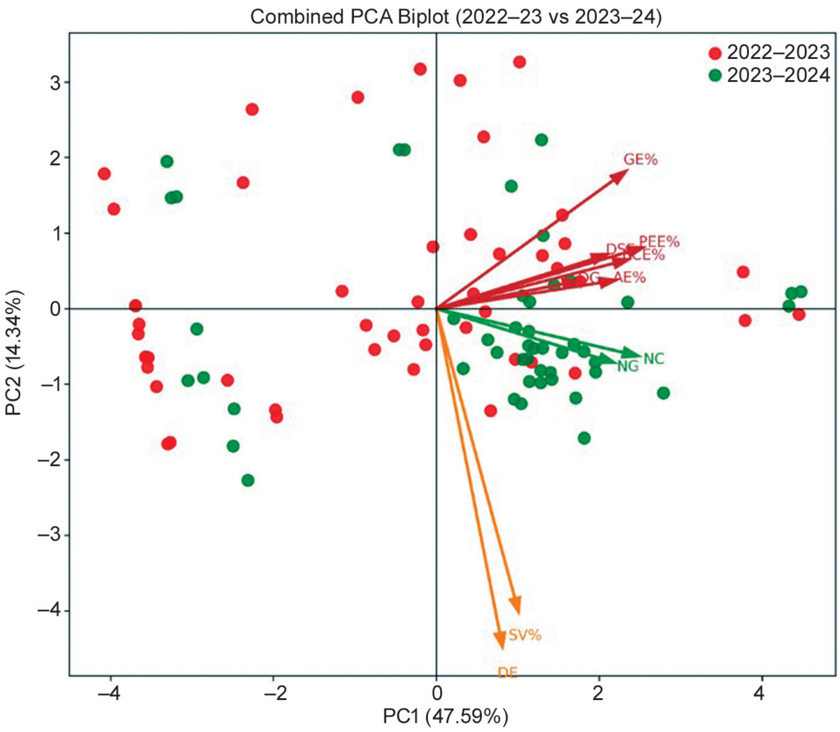

Fig. 2 Combined PCA biplot showing treatments across 2022–23 (Red) and 2023–24 (Green).
SV%, Survival%; DE, Days to embryogenesis; DSE, Direct somatic embryogenesis%; NC, No of cotyledonary embryos; NG, Number of globular embryos; DG, Days to germination; GE%, Germination efficiency%; BCE%, Bipolar conversion efficiency%; PEE, Plantlet establishment efficiency and AE%, Acclimatization efficiency %.

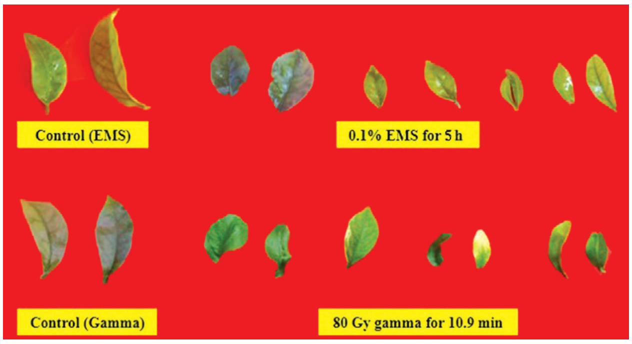

Fig. 3 Leaf variability of *in vitro* regenerants.

in 2023–24, it showed tighter clustering and greater stability of responses compared with the more dispersed pattern observed in 2022–23. Overall, the PCA confirmed a strong inverse relationship between regeneration efficiency and delayed embryogenesis/ survival stress, and highlighted year dependent modulation of mutagenic effects without altering the underlying trait associations. Similar year to year and seasonal shifts in regeneration efficiency have been reported in grapes and apple (Bisht *et al.* 2024, Carra *et al.* 2024).

Phenotypic assessment of M1 putative mutant plants: Gamma irradiation and EMS treatments significantly influenced the morphological traits of regenerants (Table 1). Plant height was drastically reduced in both mutagen treatment, declining from 5.00 cm in the EMS control to 2.46 cm under 0.1% EMS, and from 6.00 cm in the gamma control to 3.42 cm under 80 Gy. Such reductions in plant stature are common mutagenic effects, often attributed to inhibition of cell elongation and division (Bhojwani and Razdan 1996).

Internodal length also showed significant reduction, especially in EMS treated plants (0.137 cm) compared to the control (1.00 cm), while gamma exposure reduced it to 0.53 cm. The marked suppression of internode elongation under EMS may be linked to its ability to induce point mutations affecting auxin and gibberellin pathways (Van Harten 1998).

Leaf dimensions were negatively affected by both mutagens (Fig. 3). Leaf length declined from 2.93 cm (control) to 1.64 cm under EMS and 2.20 cm under gamma, while leaf width decreased from 2.05 cm to 1.24 cm and 1.01 cm, respectively. Reduced leaf expansion under mutagenic stress has been reported in cassava (Apio *et al.* 2024) and Musa (Riyadi and Sumaryono 2017), supporting our observations.

Interestingly, the number of leaves and total leaf area did not differ significantly among the treatments, indicating that mutagenic stress predominantly affected leaf expansion and elongation rather than the initiation of new leaves. This observation is consistent with the findings of Konzak *et al.* (1965). In contrast, leaf fresh weight was markedly influenced, with gamma irradiated regenerants recording a sharp decline (0.11 g) compared with the control. EMS treated plants also showed reduced fresh biomass (0.010 g), although the difference from their control was not statistically significant. Similar reductions in leaf biomass under mutagenic stress have been reported in coffee (Nkurunziza *et al.* 2025) and grapes (Ghasemi-Soloklui *et al.* 2023) supporting the current findings. This aligns with the studies of Maluszynski *et al.* (2000) which suggested that EMS causes more pronounced physiological disturbances than gamma irradiation.

Correlation studies: Correlation patterns differed between EMS and gamma irradiation, pointing to their contrasting impact on plant growth (Fig. 4). In EMS treatment, leaf area showed a strong positive correlation with fresh weight (r = 0.608), while leaf width was positively related to leaf area (r = 0.407). Plant height exhibited only weak connections with other traits, implying that EMS mainly influenced leaf development rather than overall plant height. Similar positive associations between vegetative growth traits and biomass have been reported in tomato

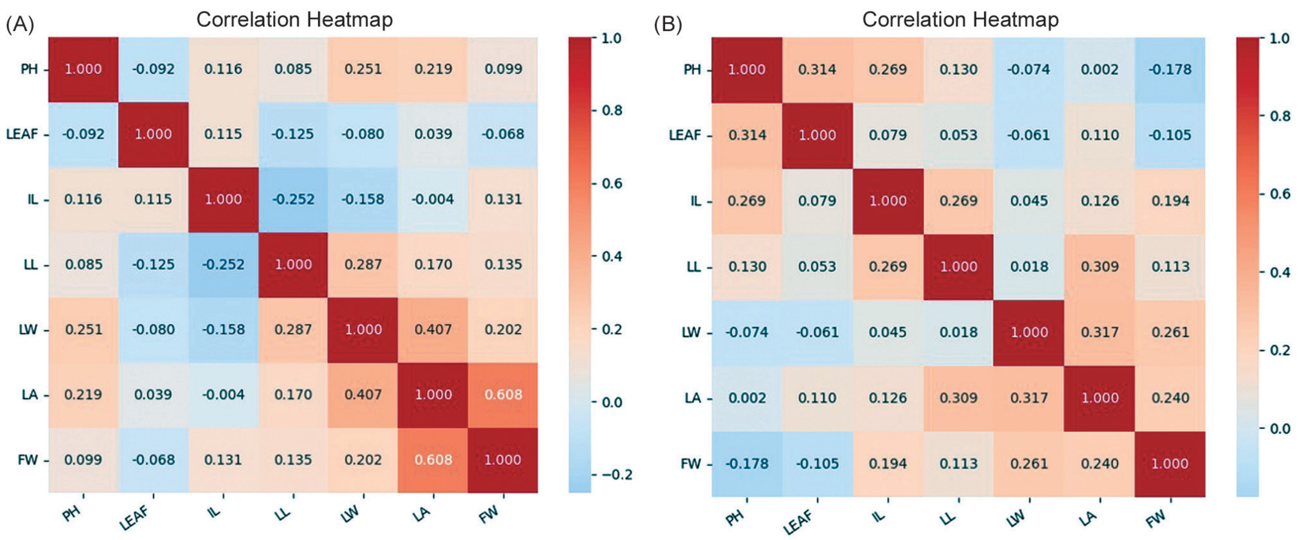

Fig. 4 Heatmap showing interrelationship among morphological parameters of A) EMS regenerants, B) gamma regenerants. PH, Plant height; LEAF, Leaf number; IL, Internodal length; LL, Leaf length; LW, Leaf width; LA, Leaf area and FW, Fresh weight.

Table 1 Effect of 0.1% EMS (5 h) and 80 Gy gamma on morphological characters of regenerated plants

Treatments	Plant height	No. of leaves	Internodal length	Leaf length	Leaf width	Leaf area	Fresh weight
Control (EMS)	5.00[a]	9.33[a]	1.000[b]	2.93[b]	2.05[b]	2.23[a]	0.027[a]
0.1% EMS for 5 h	2.46[b]	5.37[a]	0.137[a]	1.64[a]	1.24[a]	1.45[a]	0.010[a]
Control (Gamma)	6.00[a]	8.00[a]	1.000[a]	2.93[a]	2.05[a]	2.23[a]	0.078[a]
80 Gy for 20 min	3.42[b]	7.33[a]	0.530[a]	2.20[a]	1.01[b]	1.05[a]	0.011[b]
SEM ±	0.15	0.35	0.002	0.05	0.06	0.05	0.001
CD (p=0.05)	0.51	1.16	0.006	0.17	0.18	0.16	0.003
CV (%)	6.29	8.11	0.433	3.68	6.07	4.79	5.620

Values represent SEM ± . Means within a column differed followed by different letters are significantly different at ≤ 0.05 according to Tukey's HSD test.

mutants, where leaf dimensions were significantly correlated with yield attributes (Elnahal *et al.* 2025).

Under gamma irradiation, plant height correlated positively with number of leaves (r = 0.314) and internodal length (r = 0.269), indicating that taller plants produce more leaves with longer internodes. Fresh weight however, exhibited slight negative associations with plant height (r = -0.178) and leaf number (r = -0.105), while it was more closely related to leaf width (r = 0.261) and leaf area (r = 0.240), emphasizing the role of leaf expansion over plant height in determining biomass under mutagenic stress. Similar radiation-induced alterations in the relationships among plant height, leaf area, and fresh weight have also been reported in fig (Ghasemi-Soloklui *et al.* 2025).

To our knowledge, this is the first report showing contrasting correlation patterns, where biomass accumulation under EMS was mainly driven by leaf expansion, while gamma irradiation weakened association between stature related traits and biomass.

Hierarchical cluster analysis: Cluster analysis grouped 54 EMS and 56 gamma genotypes into five clusters (Fig. 5, Supplementary Table 1–2). In EMS the widest separation occurred between cluster 1 and V (5.159), while the closest association was between cluster II and III (1.758). In contrast, gamma radiation produced a much broader range, with maximum divergence between Cluster III and IV (10.00) and minimum distance between cluster IV and V (1.806). Overall, EMS treatment showed moderate variability, whereas gamma irradiation induced greater genetic divergence among the clusters.

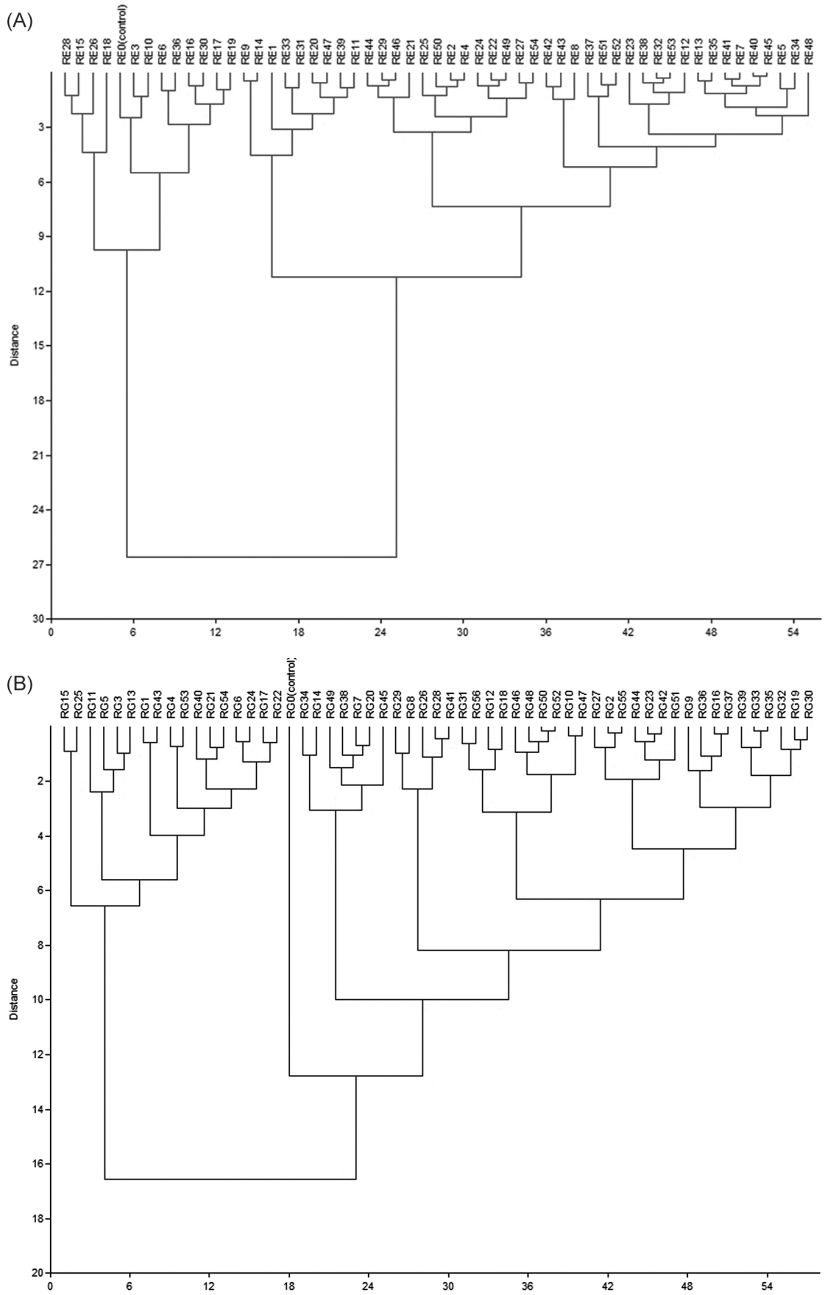

Fig. 5 Hierarchical cluster analysis of regenerants based on morphological traits. A) cluster grouping under EMS, B) cluster grouping under gamma.

Based on the findings of the present study, it is evident that the protocol can be taken forward for generating variability and developing solid mutants. Further molecular characterisation and flow cytometry would pave way towards the confirmation of mutants which could be utilised to develop varieties for traits of interest such as low seediness and climate resilience.

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