Indian Journal of Agricultural Sciences **94** (6): 644–649, June 2024/Article
https://doi.org/10.56093/ijas.v94i6.145347

EMS induced *in vitro* mutagenesis for solid mutant induction and validation of mutants using morphological and molecular tools in kinnow mandarin (*Citrus nobilis* × *Citrus deliciosa*)

THEIVANAI MURUGAN[1], OM PRAKASH AWASTHI[1]*, BHARADWAJ CHELLAPILLA[1] and KRIPA SHANKAR[1]

ICAR-Indian Agricultural Research Institute, New Delhi 110 012, India

Received: 16 November 2023; Accepted: 13 March 2024

ABSTRACT

Non-chimeric regeneration via *in vitro* mutagenesis is one of the niche areas in perennial fruit crops to shorten the breeding cycle. However, the recovery of M_1 population is a major hurdle due to lack of efficient protocols. Hence the present experiment was conducted during 2021–23 at ICAR-Indian Agricultural Research Institute, New Delhi on EMS induced *in vitro* mutagenesis and its validation in kinnow mandarin (*Citrus nobilis* Loureiro × *Citrus deliciosa* Tenora). The specialized direct somatic embryogenesis (DSE) protocol was standardized and the optimized explants (*in ovulo* nucellus) were treated with 3 EMS concentration of 0.1, 0.5 and 1.0% for 1, 3 and 5 h. Based on explant survival, probit analysis was calculated and results revealed that the LD_{50} for *in ovulo* nucellus explants was 0.3% for 5 h. For a dose rate higher than LD_{50} i.e. E_8 (0.5% for 5 h) and as compared to control, the embryogenesis efficiency was reduced to 65%, likewise embryo production (23.64%), germination (46.15%), conversion (22.76%), establishment (25.85%) and acclimatization (19.44%) showed reducing trend. The EMS derived M_1 population showed variability both at morphological and molecular level. Among the markers tested, random amplification of polymorphic DNA (RAPD) and simple sequence repeats (SSR) could demarcate the mutants from mother plant. The origin of homohistont was confirmed from the morphology of observed chlorophyll defective mutants. Thus the optimized EMS dose using DSE system can be effectively used for production of trait specific solid mutants and the morphological and molecular screening protocols have practical value in early selection of M_1 population.

Keywords: Direct somatic embryogenesis, EMS, *In vitro* mutagenesis, Molecular validation, PCA, Phenomics

Citrus (2n=2x=18; 1C=320.50 Mb) commercially grown in more than 140 countries between 40 ˚N and 40 ˚S latitudes in an area of 13.25 million ha with a production of 208.47 million tonnes (FAO 2023). In India after mango and banana, citrus occupies third position with an area of 1.10 million ha and a production of 14.85 million metric tonnes (Anonymous 2022). Among citrus group, kinnow an inter-specific mandarin hybrid of *Citrus nobilis* Loureiro × *Citrus deliciosa* Tenora has revolutionized the citrus industry of India because of its wider adaptability, attractive golden yellow peel, higher juice recovery, quality and affordable market price (Kumar *et al.* 2021). This golden fruit also has the potential for juice processing provided low seeded kinnow mandarin (<10 seeds/fruit) are developed compared to 30–35 seeds/fruit (Kumar *et al.* 2021).

[1]ICAR-Indian Agricultural Research Institute, New Delhi.
*Corresponding author email: awasthiciah@yahoo.com

Mutagenesis has been widely used as breeding tool for trait specific varietal improvement in citrus (Roose and Williams 2007). Current knowledge on protocol refinement for woody perennials is scanty (Predieri 2001). In this direction evolutionary adaptive behaviour of perennial woody plants i.e., the long juvenility, self-incompatibility, sterility etc., implicate the use of single cell targeted mutagenesis for solid mutant induction through cellular totipontecy mediated *in vitro* mutagenesis (Predieri 2001, Roose and Williams 2007). In this direction chemical mutagens such as EMS (Ethyl methanesulfonate) have an advantage because of its ability to create point mutations (single G/C base pair changes) (Talebi *et al.* 2012).

The *in-vitro* mutagenic effect of EMS has been tested only in *Citrus jambhiri* (Lush.) and *C. sinensis* (L.) Osbeck (Ge *et al.* 2015, Savita *et al.* 2017) however, with a poor plantlet recovery. Further no attempt has so far been made on EMS induced *in vitro* mutagenesis for solid mutant induction in mandarin which also requires reliable and reproducible cellular totipotency mediated high frequency

plantlet recovery techniques. Thus, the *in ovulo* nucellus based specialized DSE system (Murugan *et al.* 2023) which could ensure true to the type regeneration (removal of zygote) and solid mutant induction (from single cells of nucellus) by removal of pre-existing embryos from the initial explant has been standardized but the mutagenesis ability of the same needs to be tested. Hence the present investigation was therefore, aimed to induce solid M_1 mutant population using specialized (*in ovulo* nucellus based) high frequency DSE protocol and further validation for its morphological and genetic changes using phenomics and molecular markers in kinnow mandarin.

MATERIALS AND METHODS

Plant material: The present experiment was conducted during 2021–23 at ICAR-Indian Agricultural Research Institute (28° 38′ 23″ N, 77° 09′ 27″ E), New Delhi for which a 7-year old mother plant of kinnow mandarin (grafted on rough lemon rootstock) maintained at a spacing of 6 m × 6 m was used as a source of explants.

Explant preparation for EMS treatment: As per the standardized protocol, ovules of >4 mm were freshly extracted from surface sterilized 21–22 mm sized fruits (2nd week of May to 1st week of June) and taken in sterile petri dish inside the laminar air flow (LAF) hood. To prevent dehydration, Petri dishes containing tissue paper were moistened with sterile distilled water. The collected ovules were pooled and uniform sized ovules were selected for EMS treatment during 2021–22.

EMS treatment: The ovules were treated with different doses of EMS concentrations (0.1, 0.5 and 1.0%) and duration of 1, 3 and 5 h at the Central Tissue Culture Laboratory of ICAR-National Institute for Plant Biotechnology, New Delhi. For each concentration 150 ml conical flask containing double the quantity of sterile EMS solution as that of explant with *p*H 6.8–7.0 was used in three replicates. The explants required for three different durations i.e. ~100 ovules/concentration (~30 ovules/duration/concentration) were added in respective conical flask containing EMS solution (EMS mixed in sterile distilled water) under LAF and then tightly cotton plugged and sealed with parafilm®. The explants were then agitated at 120 rpm using orbital shaker cum incubator for uniform EMS exposure. Thereafter explants (~30 ovules/concentration) were taken out after 1 h from the respective flask of each concentration and the rest maintained aseptically on a shaker for subsequent studies at varying duration of 3 and 5 h. To remove the excessive EMS solution, the explants were rinsed repeatedly 5 times (5–10 min/rinse) with sterile distilled water.

Post handling procedures of EMS treated explants: Immediately after washing 25 *in ovulo* nucellus explants from each EMS treatment (0.1, 0.5 and 1.0%) of 1, 3 and 5 h duration in three replicates were prepared from treated ovules as outlined by Murugan *et al.* (2023). The *in ovulo* explants were inoculated on somatic embryogenesis induction medium and incubated under dark in culture room with 75 × 80% relative humidity and 25 ± 2° temperature.

LD_{50} determination: At the time of first subculture i.e. 60 days after post EMS treatment, lethal dose (LD_{50}) was determined by counting explant survival using the formula given as:

$$\text{DSE - Survival \%} = \frac{\text{Number of survived explant}}{\text{Number of EMS treated explants cultured}} \times 100$$

Mutant regeneration: As outlined below the mutants were regenerated using standard protocols outlined by Murugan *et al.* (2023). Accordingly, the embryogenesis ability of EMS treated *in ovulo* nucellus was assessed on induction cum maturation medium added with 5.0 mg/litre kinetin and 1000 mg/litre malt extract (ME) in basal Driver and Kuniyuki Walnut (DKW) medium. After maturation, 25 randomly selected uniform embryos/replicate were placed on germination cum conversion medium containing 2.0 mg/litre gibberellic acid (GA_3), 0.5 mg/litre ά-naphthaleneacetic acid (NAA), 100 mg/litre spermidine and 10% (v/v) coconut water (CW) in Murashige and Tucker medium (MT). Bipolar converted seedlings were transferred from dark to light on filter paper bridge in test tube containing basal liquid establishment medium. Finally, established plantlets were primary hardened in 250 ml culture bottle containing 2:1:1 composition of cocopeat: vermiculite: perlite. The timely observations on EMS effect were noted on each step of regeneration in all three replications.

$$\text{Embryogenesis efficiency (\%)} = \frac{\text{No. of survived explants initiated somatic embryos}}{\text{No of explants survived after irradiation}} \times 100$$

$$\text{Germination efficiency of DSE system (\%)} = \frac{\text{No. of embryos showing primordial initiation}}{\text{No. of embryos transferred to germination medium}} \times 100$$

$$\text{Bipolar conversion efficiency (\%)} = \frac{\text{No. of embryos initiated shoot and root growth (>3 cm)}}{\text{No. of embryos germinated}} \times 100$$

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

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

Morphological characterization of mutants: Primary hardened plants were further subjected to secondary hardening during 2022–23. Phenotypic variability induced by EMS at various doses (concentration and duration) was assessed using manual and automated methods for careful documentation of phenotypes. The phenomics platform was optimized to get digital morphological data through image

processing software for future references. Randomly selected hardened plants of control and E_8 M_1 populations were imaged from side view at the angle of 0° and 90°along with top view. Subsequently, these were analyzed to get digital data using Lemna grid software (LemnaTec Scanalyzer3D high throughput phenotyping platform, Achen, Germany).

Molecular validation of mutants: Genomic DNA of morphologically discriminated M_1 mutants along with mother plants was isolated from secondary hardened plants and quantified using Murugan *et al.* (2023) protocol. Edible mandarin cultivars specific 22 identified highly polymorphic molecular markers, viz. RAPD (11), SSR (3) and ISSR (8) of dominant and co-dominant nature were used for validation of genetic variability created by EMS using Pal *et al.* (2013), Kumar *et al.* (2020) and Murugan *et al.* (2023) protocols, respectively. For conformity experiment was replicated for three times.

Statistical analysis: The LD_{50} was calculated using probit analysis using SPSS software. One-way- ANOVA with completely randomized design (CRD) was used to test the effect of EMS on embryogenesis. To analyze the statistical significance of *in vitro* studies and multiple variants (PCA) RStudio, Version 4.2.0 (RStudio Inc, Boston, MA, USA) was used and Duncan's multiple range test (DMRT) was used to differentiate the mean at $P<0.05$. Percentage data were arc sine transformed before subjected to ANOVA.

RESULTS AND DISCUSSION

LD_{50} determination: In the present investigation per cent survival decreased with increasing doses of EMS concentration. Probit analysis revealed the 50% mortality at 0.31% concentration and 5 h duration (Fig. 1). However, complete mortality of treated explants was observed in E_9 (1.0% EMS concentration and 5 h duration) (Table 1). The decrease in explant survival observed in the present investigation confirms the efficiency of the EMS treatment methods on altering the explant physiology, damage caused to the DNA and other genetic material. The EMS induced reduction in survival implicates the amenability of cells to changes either at matured or immature state. The 50% lethal effect of 0.3% EMS concentration and 5 h treatment duration of *in-ovulo* nucellus are in consonance with the study reports of Devi *et al.* (2021) in acid lime.

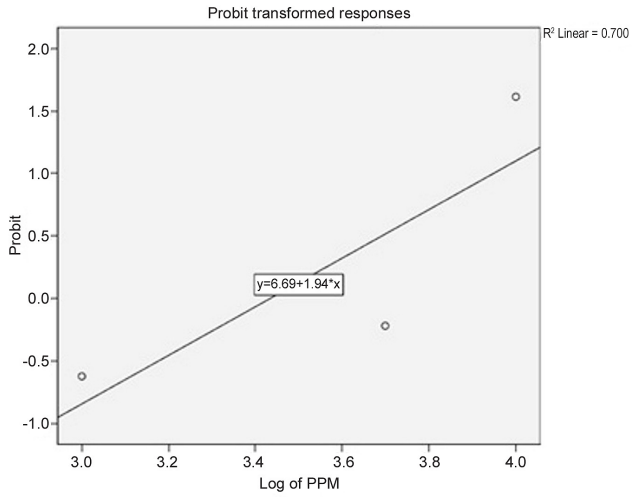

Fig. 1 Probit analysis and LD_{50} for EMS treated direct somatic embryogenesis system *in ovulo* nucellus explants in kinnow mandarin.

EMS influence on somatic embryogenesis: Increase in EMS concentration and treatment duration linearly reduced the embryogenesis efficiency. Among the treatment E_8 (0.5% for 5 h) i.e., the dose rate next to LD_{50} embryogenesis was reduced to 65.51% in comparison to control. Days to embryogenesis was not affected at lower concentrations (0.1–0.5%) and treatment durations of 1–3 h (E_0–E_5) but it was delayed at higher concentration of 1.0% when exposed for 3 and 5 h. Embryogenesis was delayed by 18.92 days in E_8 (Table 1) while embryo production was reduced by 23.29% in the same treatment. Reduction in embryogenesis efficiency, embryo production capacity and delayed embryogenesis with increasing EMS concentration explains the guanine alkylating effect and its subsequent cell cycle arrest for cellular repair process and strong point mutagenic effect on widespread quinine nucleotides in the chromosome. Similar results on reduced embryogenesis efficiency and embryo production have been reported in herbaceous perennials when organized tissue was used as an explant (Nasri *et al.* 2021).

Effect of EMS on germination, conversion, establishment and acclimatization: Germination was highly inhibited in the E_8 and it was reduced by 46.12% in divergence to control. However, the varying EMS doses did not impart any significant influence on days to germination but the bipolar conversion efficiency was reduced to 22.76% in E_8. Although the establishment per cent was reduced to 25.85%, the treatment effect of E_8 on plantlet acclimatization was merely 19.44% (Table 1). Inverse effect of ascending EMS doses on delay in germination, reduced germination and conversion efficiency suggests EMS induced alteration in somatic embryos which hindered the normal developmental process as a consequence of altered cellular differentiation and arrangements. The germination related effect was however moderate when compared to the detrimental effect reported by other researchers on seed germination of few citrus species (Jawaharlal *et al.* 1992, Devi *et al.* 2021). Reduction in establishment and acclimatization efficiency observed with ascending doses of EMS concentration shows the effect of chemical mutagen on developmental events of root and shoots morphogenesis. The better establishment, acclimatization and high mutant population recovery of EMS induced mutants gives insight about the elimination of deleterious mutants at various level of regeneration step. This can be correlated with the speculations on effect of EMS on cytochrome oxidase content, reduced respiration thus leading to the developmental disparities. The EMS induced reduction in seedling establishment has also been previously observed in acid lime, rough lemon seeds (Jawaharlal *et al.* 1992, Kaur *et al.* 2010).

Growth related parameters: The mean shoot length,

Table 1 Effect of EMS doses on various steps of *in vitro* direct somatic embryogenesis mediated regeneration in kinnow mandarin

EMS treatment	Per cent survival (%)	Embryo-genesis efficiency (%)	Days to embryo-genesis	Embryo production capacity	Germination efficiency (%)	Days to germination	Bipolar conversion efficiency (%)	Plantlet establishment efficiency (%)	Acclimti-zation efficiency (%)
E_0 (control)	94.67 (76.69)^{a}	36.65 (37.28)^{a}	54.41^{e}	110.58^{c}	86.67 (68.62)^{a}	11.36^{b}	75.32 (60.25)^{a}	61.81 (51.85)^{a}	100.00 (90.05)^{a}
E_1 (0.1% 1 h)	92.00 (73.61)^{a}	27.51 (31.65)^{b}	55.21^{e}	111.30^{c}	85.33 (67.52)^{a}	11.25^{b}	74.96 (60.01)^{a}	58.68 (50.03)^{a}	100.00 (90.05)^{a}
E_2 (0.5% 1 h)	81.33 (64.43)^{b}	24.52 (29.70)^{b}	54.74^{e}	113.60^{bc}	84.00 (66.46)^{a}	11.99^{ab}	74.86 (59.93)^{a}	57.36 (49.26)^{a}	100.00 (90.05)^{a}
E_3 (1.0% 1 h)	77.33 (61.60)^{b}	24.21 (29.49)^{b}	55.55^{e}	116.23^{b}	78.67 (62.52)^{a}	11.43^{b}	74.69 (59.82)^{a}	59.21 (50.33)^{a}	100.00 (90.05)^{a}
E_4 (0.1% 3 h)	72.00 (58.08)^{c}	22.06 (28.03)^{bc}	54.23^{e}	121.53^{a}	81.33 (64.43)^{a}	11.83^{ab}	72.01 (58.09)^{a}	61.01 (51.39)^{a}	100.00 (90.05)^{a}
E_5 (0.5% 3 h)	65.33 (53.96)^{d}	16.42 (23.92)^{cd}	59.39^{d}	123.83^{a}	65.33 (53.96)^{b}	12.35^{a}	65.62 (54.13)^{ab}	54.04 (47.34)^{a}	82.22 (65.10)^{b}
E_6 (1.0% 3 h)	42.67 (40.80)^{f}	9.44 (17.91)^{e}	81.00^{a}	69.67^{f}	38.67 (38.47)^{d}	11.74^{ab}	48.48 (44.15)^{c}	50.00 (45.02)^{a}	72.22 (58.22)^{b}
E_7 (0.1% 5 h)	68.00 (55.58)^{d}	15.60 (23.28)^{d}	65.22^{c}	95.67^{d}	52.00 (46.17)^{c}	11.48^{ab}	71.49 (57.76)^{a}	54.50 (47.61)^{a}	86.67 (68.62)^{ab}
E_8 (0.5% 5 h)	53.33 (46.94)^{e}	12.64 (20.83)^{de}	73.33^{b}	84.83^{e}	46.67 (43.11)^{cd}	11.89^{ab}	58.18 (49.73)^{bc}	45.83 (42.63)^{a}	80.56 (63.87)^{b}
E_9 (1.0% 5 h)	0.00 (0.00)^{g}	0.00 (0.00)^{f}	0.00^{f}	0.00^{g}	0.00 (0.00)^{e}	0.00^{c}	0.00 (0.00)^{d}	0.00 (0.00)^{a}	0.00 (0.00)^{c}
Mean	64.67	18.91	55.31	94.72	61.87	10.53	61.56	50.24	82.17
LSD (P>0.05)	4.57	4.25	2.19	4.72	7.31	0.90	6.91	9.39	17.48

Values in the parenthesis are arc sine transformed. Mean followed by alphabets in column represent the statistical significance.

root length and number of leaves/plant decreased with increasing dose of EMS. Complete albino plants and plants containing all the leaves with similar mosaic pattern confirms the regeneration of plants from single mutated cell and thus fulfill the objectives of solid mutant induction. The defects in shoot formation (root only conversion), root formation (shoot only conversion), reduction/increase in leaf size or leave thickness or shoot/root were some of the abnormalities observed among the mutants. To assess the efficiency of Phenomics platform on mutant discrimination the digital data was obtained from selective mutants (Fig. 2). The obtained data reflected its easiness in mutant discrimination. Morphological abnormalities in E_8 explain the EMS induced changes in hormonal biosynthesis pathways. Severe root abnormalities observed in the present study than shoot abnormalities was possibly the effect of EMS on auxin biosynthesis. Auxin biosynthesis alterations effect on EMS as observed in present study are in line with the observations of Mohapatra *et al.* (2014) and Nasri *et al.* (2021) in rice and chrysanthemum. Chlorophyll abnormality was comparatively higher in M_1 EMS population and can be attributed to chloroplast organelle damage. Similar effect on chlorophyll abnormalities have also been reported in banana (Bidabadi *et al.* 2011), rice (Viana *et al.* 2019) and other annuals (Gnanamurthy and Dhanavel 2014, Deepti and Remesh 2016, Nasri *et al.* 2021).

Principal component analysis: To identify the most important factor which was influenced by EMS treatment and for future *in vitro* mutagenesis studies, the PCA analysis was carried out. The PCA of somatic embryogenesis related

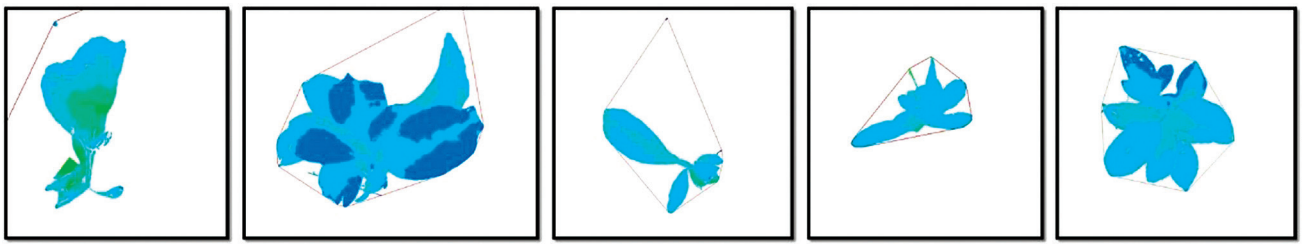

Fig. 2 Phenomics images of EMS variants in kinnow mandarin.

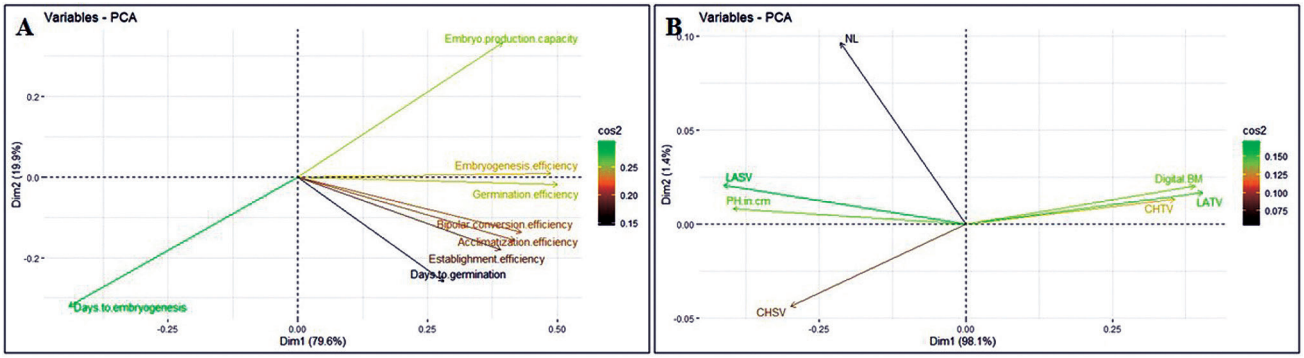

Fig. 3 Principle component analysis (PCA) with somatic embryogenesis and phenomics digital data related parameters in kinnow mandarin. (A) Plot representing somatic embryogenesis related variables; (B) Plot representing phenomics related variables.

variables exhibited 79.6 and 19.9% variation in the first and second component. Somatic embryogenesis efficiency and embryo production capacity were observed to be the important variables for EMS induced *in vitro* mutagenesis (Fig. 3). PCA for morphological parameters registered 98.1 and 1.4% variation in the first two principal components. The significant variables of the first components include variables such as convex hull top-view, leaf area top-view and digital biomass (Fig. 3). Literature related to PCA analysis on *in vitro* mutagenesis is scanty. However, the efficiency of PCA on assessing the mutant phenotypes has been reported in lentil (Debnath *et al.* 2022).

Demarcation of mutants using molecular marker: The amplified banding pattern related scoring data of 22 markers revealed that except control all the regenerants obtained after selective EMS treatments were of mutants (Table 2). Among the markers tested the RAPD and SSR showed polymorphism in banding pattern, while ISSR showed monomorphism. The variable number of bands and position revealed that the genetic changes occurred due to mutation. Molecular characterization although showed variability, only RAPD and SSR markers could detect the variation from *in vitro* derived M_1 population. The EMS induced point mutations and resulted frameshifts in the coding and non-coding regions may be the reason for changes observed amongst the individuals. Earlier studies on demarcation of variations from *in vitro* derived M_1 population using RAPD and SSR have not been reported in citrus, but have proved its robustness in crop plants (Sharma *et al.* 2022).

From the detailed investigation it can be concluded that the direct somatic embryogenesis protocol tested for its efficiency on EMS induced *in vitro* mutagenesis have practical utility in inducing solid mutant. The optimized dose can induce variability and also simultaneously can support higher recovery of mutant individuals. The morphological and molecular validation protocol tested in the present study shows its efficiency on distinguishing mutants from the mother plant. Hence the present protocol can be effectively used for trait specific improvement in kinnow mandarin.

Table 2 Molecular validation of *in vitro* EMS induced M_1 mutants in kinnow mandarin

Molecular marker	Randomly selected morphologically different M_1 Kinnow mutants					
	Mother plant	EM_1	EM_2	EM_3	EM_4	EM_5
RAPD						
OPA1	10	10	10	10	10	10
OPA 2	4	4	4	4	4	4
OPA 4	7	9	9	8	0	0
OPA 6	7	5	5	5	5	5
OPA 8	11	11	11	11	8	0
OPA 9	7	8	8	9	9	9
OPA 10	9	9	5	9	9	8
OPA 11	9	9	2	8	6	0
OPA 13	9	9	9	9	9	9
OPA 17	11	11	7	11	11	10
OPA 18	7	5	4	4	5	6
ISSR						
UBC 807	7	7	7	7	7	7
UBC 808	6	6	6	6	6	6
UBC 812	8	8	8	0	8	8
UBC 825	10	10	10	10	10	10
UBC 827	6	6	6	6	6	6
UBC 841	9	9	9	0	9	9
UBC 855	6	6	6	6	6	6
UBC 864	3	3	3	3	3	3
SSR						
CCSM 13	4	4	4	4	4	4
CCSM 40	1	1	1	0	0	1
AMB2	1	1	1	1	2	1
Total	152	151	135	131	137	122

ACKNOWLEDGEMENT

The authors are grateful to the ICAR-Indian Agricultural Research Institute, New Delhi, for providing the financial support in the form of Senior Research Fellowship to carry out this study.

REFRENCES

Anonymous. 2022. Second advanced estimate of area and production of horticultural crops. Ministry of Agriculture and Farmers Welfare, Government of India.

Bidabadi S S, Mahmood M, Meon S, Wahab Z and Ghobadi C. 2011. Evaluation of *in vitro* water stress tolerance among EMS induced variants of banana (*Musa* spp., AAA), using morphological, physiological and molecular traits. *Journal of Crop Science and Biotechnology* **14**(4): 255–63.

Debnath S, Sarkar A, Perveen K, Bukhari N A, Kesari K K, Verma A, Chakraborty N R and Tesema M. 2022. Principal component and path analysis for trait selection based on the assessment of diverse lentil populations developed by gamma-irradiated physical mutation. *BioMed Research International* 1–14.

Deepti T and Remesh K N. 2016. Impact of EMS induction on morphological, anatomical and physiological traits of bhendi [*Abelmoschus esculentus* (L.) Moench]. *International Journal of Recent Research in Life Science* **3**(2): 4–11.

Devi M D, Kumar C S R, Rajangam J, Santha S and Sankar C. 2021. Determination of lethal dose (LD_{50}) and effect of physical and chemical mutagenesis in acid lime var. PKM 1. *The Pharma Innovation Journal* **10**(11): 583–88.

FAO. 2023. https://www.fao.org/faostat/en/#data/QCL

Ge H, Li Y, Fu H, Long G, Luo L, Li R and Deng Z. 2015. Production of sweet orange somaclones tolerant to citrus canker disease by *in vitro* mutagenesis with EMS. *Plant Cell, Tissue and Organ Culture* **123**: 29–38.

Gnanamurthy S and Dhanavel D. 2014. Effect of EMS on induced morphological mutants and chromosomal variation in cowpea (*Vigna unguiculata* L.). *International Letters of Natural Sciences* **22**: 33–43.

Jawaharlal M, Sambandamorthy S and Irulappan I. 1992. Effect of gamma ray and EMS on seed germination and seedling growth in acid lime (*Citrus aurantifolia* Swingle). *South Indian Horticulture* **369**: 332–36.

Kaur S and Rattanpal H S. 2010. Effect of mutagens on *in-vitro* seed germination and growth of rough lemon (*Citrus jambhiri* Lush.) seedlings. *The Indian Journal of Agricultural Sciences* **80**(9): 773–76.

Kumar S. 2020. 'Morphogenetic characterization of Kinnow mutants'. PhD Thesis, ICAR-India Agricultural Research Institute, New Delhi, India.

Kumar S, Awasthi O P, Sharma R M and Pradhan S. 2021. Physiological and biochemical responses of Kinnow mandarin (*Citrus nobilis* × *Citrus deliciosa*) to EMS induced mutagenesis. *The Indian Journal of Agricultural Sciences* **91**: 1015–019.

Mohapatra T, Robin S, Sarla N, Sheshasayee M, Singh A K and Singh K. 2014. EMS induced mutants of upland rice variety Nagina22: Generation and characterization. *Proceedings of The Indian National Science Academy* **80**: 163.

Murugan T, Awasthi O P, Singh S K, Chawla G, Solanke A U, Kumar S and Jha G K. 2023. Molecular and histological validation of modified *in ovulo* nucellus culture based high-competency direct somatic embryogenesis and amplitude true-to-the-type plantlet recovery in Kinnow mandarin. *Frontiers in Plant Science* **14**: 1–17.

Nasri F, Zakizadeh H, VAfaee Y and Mozafari A A. 2021. *In-vitro* mutagenesis of chrysanthemum morifolium cultivars using ethylmethanesulphonate by ISSR and IRAP markers. *Plant Cell Tissue and Organ Culture* **149**: 657–73.

Pal D, Malik S K, Kumar S, Choudhary R, Sharma K C and Chaudhury R. 2013. Genetic variability and relationship studies of mandarin (*Citrus reticulata* Blanco) using morphological and molecular markers. *Agricultural Research* **2**: 236–45.

Predieri S. 2001. Mutation induction and tissue culture in improving fruits. *Plant cell, Tissue and Organ culture* **64**(2–3): 185–210.

Roose M L and Williams T E. 2007. *Mutation Breeding*, pp. 345–52. Khan I A (Ed). Wallingford, CABI International.

Savita P P K, Virk G S and Nagpal A. 2017. *In-vitro* selection of resistant/tolerant mutant lines of *Citrus jambhiri* Lush. using crude culture filtrate of *Phytophthora parasitica* and their randomly amplified polymorphic DNA analysis. *Journal of Phytopathology* **165**(11–12): 771–81.

Sharma P, Nath A K, Dhiman S R, Dogra S and Sharma V. 2022. Characterization of carnation (*Dianthus caryophyllus* L.) genotypes and gamma irradiated mutants using RAPD, ISSR and SSR markers. *South African Journal of Botany* **148**: 67–77.

Talebi A B, Talebi A B and Shahrokhifar B. 2012. Ethyl methane sulphonate (EMS) induced mutagenesis in malaysian rice (cv. MR219) for lethal dose determination. *American Journal of Plant Science* **3**: 1661–665.

Viana V E, Pegoraro C, Busanello C and Oliveira A C. 2019. Mutagenesis in rice: The basis for breeding a new super plant. *Frontiers in Plant Science* **10**: 1–28.