



Physiological and biochemical responses of Kinnow mandarin (*Citrus nobilis* × *Citrus deliciosa*) to EMS induced mutagenesis

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ABSTRACT

The present study was carried out to enhance the genetic variability in Kinnow mandarin (*Citrus nobilis* Loureiro × *Citrus deliciosa* Tenora) using ethyl methanesulphonate (EMS) during 2016–17 and 2017–18. Plants created with different doses of EMS concentration, viz. 0.05%, 0.1%, 0.2% and 0.5% were examined for the various physiological and biochemical alterations in putative mutants aged six years and compared with the wild type (WT). A dose-dependent decrease in leaf area was recorded in the mutants with increasing doses of EMS concentration and as compared to WT, maximum reduction of 135.52 and 104.92% was witnessed in the mutants E-19 and E-17 developed from 0.5% EMS, whereas the leaves were more succulent in E-16. Chlorophyll *a* content was higher than chlorophyll *b* in the mutated population. As compared to WT, maximum decrease in chlorophyll *a*, total chlorophyll was noticed in the mutants E-19 generated from 0.5% EMS. Contrary to the physiological alterations, the total phenol, proline and total protein content in the mutants were upregulated by nearly two fold in the mutants developed beyond 0.1% EMS. Significant increase was also noticed in the antioxidant enzymes SOD, CAT, POX and GR activity in the mutants E-16 to E-20 induced from 0.5% EMS. The variation in traits of interest observed in the present study such as better leaf succulency and upregulated antioxidant enzyme activity in the mutant E-16 (0.5% EMS) suggests that the developed mutant could be utilised in Kinnow improvement for abiotic stress tolerance.

Keywords: Antioxidant enzymes, Chlorophyll, EMS, Kinnow mutants, Leaf area, Phenol

Kinnow mandarin (*Citrus nobilis* Loureiro × *Citrus deliciosa* Tenora) is a preferred fruit crop by the orchardist of the arid and semi-arid regions of the country. This beautiful golden orange mandarin has revolutionised the citrus industry in these regions owing to its prolific bearing habit, higher yield, better economic return and consumer's preference. Besides fruit, the demand of Kinnow juice in the recent past has also increased because of its high juice recovery per cent. The processing sector, however, could not make much dent in this direction because of certain intrinsic trait, i.e. the high number of seeds (30-35 seeds/fruit) which causes bitterness in the juice due to crushing of seeds (Kumar *et al.* 2019). The crop, therefore, needs to be improved for certain traits such as the low number of seeds/fruit (<10) as compared to 30-35 seeds/fruit, dwarf statured plant, fruit maturity, quality attributes etc.

Varietal improvement in citrus through conventional breeding had been rather slow because of several inherent biological factors. Facultative apomixis, self and cross-

incompatibility, long juvenile period and high heterozygosis are some of the vast arrays of impediments faced by citrus breeders in conventional hybridization. Valuable mutants through chemical mutagen such as ethyl methanesulphonate (EMS) have been used as a breeding tool for creating variability and developing variety with traits of interest as it produces random point mutations in the genetic material (Predieri 2001, Dhakshanamoorthy *et al.* 2010, Mallick *et al.* 2016, Rime *et al.* 2019) through the morpho-physiological and biochemical alterations. Significant alteration in EMS induced mutants have been reported to alter leaf area, chlorophyll pigments, total phenolic, proline and protein content etc. by various researchers (Ghani *et al.* 2014, Deepthi and Ramesh 2016, Rime *et al.* 2019). The mutated plants have been reported to regulate the anti-oxidant enzymes defence system to protect themselves against the harmful effects of reactive oxygen species (ROS). Enhanced SOD, CAT, POX and GR activities in the EMS treated plants were reported in different crops. Thus in the present study, the varying effect of EMS was conducted to understand the physio-biochemical alterations in the putative mutants.

MATERIALS AND METHODS

Experimental procedure and plant material: The present experiment was conducted at the Division of

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Fruits and Horticultural Technology, ICAR-IARI, New Delhi. The experimental site (77° 12' E; 28° 40' N 228.61 masl) is characterized as semi-arid and subtropical, with hot and dry summers and cold winter. Twenty putative mutants of Kinnow mandarin developed during 2011 with varying doses of EMS concentration, i.e. 0.05% (E-1 to E-5), 0.1% (E-6 to E-10), 0.2% (E-11 to E-15) and 0.5% (E-16 to E-20) were compared with non-treated Kinnow (wild type) of the same age. The mutants and wild type (WT) were developed from the same source of bud wood, budded on *Jatti Khatti* (*Citrus jambhiri* Lush) rootstock, and field planted during 2011 at a spacing of 3m × 3m. The plants were maintained under uniform cultural practices recommended for Kinnow. The data on physiological and biochemical alterations were recorded on five plants from each treatment for two consecutive years, i.e. 2017 and 2018 and the mean of two years were computed for statistical analysis. The methods used for determining the various physiological and biochemical parameters in the present study are given below.

Physiological parameters: Leaf area (LA) was recorded using a LI 3100 area meter (Li-Cor, USA), thereafter fresh and dry mass of 10 leaves was recorded. Several indices of leaf sclerophylly were calculated by the formulae suggested by Ennajeh *et al.* (2010). Chlorophyll fractions were extracted from fresh leaves by following the method of Barnes *et al.* (1992).

Biochemical parameters: Total phenol in leaf tissue was assayed following the protocol of Malik and Singh (1980). Proline content in leaf tissue was estimated by acid ninhydrin method suggested by Bates *et al.* (1973). Proteins concentration in the leaf tissue was determined by the method given by Bradford (1976). For determining the antioxidant enzymatic activity, fresh leaves were collected in icebox to prevent the proteolytic activity. One g of cleaned sample was ground in pre-chilled mortar and pestle at 4°C in 5 mL of chilled phosphate buffer (50 mM; pH 7.0). Homogenates were centrifuged at 15000 × g for 20 min at 4°C. The supernatant stored at -20°C was used to measure the activities of superoxide dismutase (SOD), catalase (CAT), peroxidase (POX) and glutathione reductase (GR).

Statistical analyses: The experiment was conducted in a complete block design (CBD) with 4 replications. The pooled data of 2 consecutive years were subjected to analysis of variance using the SAS package (9.3 SAS Institute, INC., USA) followed by Tukey's Honest test. P values ≤ 0.05 were considered as significant.

RESULTS AND DISCUSSION

Physiological parameters

Leaf sclerophylly: Variation in leaf sclerophylly characteristics was recorded in the putative mutants (Table 1). As compared to the WT it was de-escalated by 135.52 and 104.92 in the mutants E-19 and E-17 developed from 0.5% EMS. Leaf fresh weight was higher in the mutants E-1 and E-2 (0.05%), while a significant reduction in leaf

fresh weight was noticed in the mutants E-19 and E-20 generated from 0.5% EMS. Density of foliar tissue (DFT) was more in E-12 (0.2% EMS) and less in E-8 (0.1% EMS). Leaf succulency in terms of values was indexed maximum in the mutants E-16 and E-20 created from 0.5% EMS. The increased values in the leaf sclerophylly characteristics at higher doses might be due to the stimulatory and inhibitory effect of this alkylating agent on plant morphogenesis, which was also evident from the growth data recorded on plant height and plant spread (data published, Kumar *et al.* 2020). The literature on the effect of different EMS treatments on phenological variation in leaf characteristics is scanty except those reported by Kaur and Rattanpal (2010) in rough lemon (*Citrus jambhiri* Lush) and Deepthi and Ramesh (2016) in okra (*Abelmoschus esculentus* (L.) Moench).

Photosynthetic pigments: The data generated on photosynthetic pigments showed that chlorophyll 'a' was more than chlorophyll 'b' in the EMS derived mutants (Table 1). A general decrease in chlorophyll 'a' and total chlorophyll was noticed in the mutants and the decrease was maximum in E-19 induced from 0.5% EMS. The chlorophyll 'b' content, in general, was more in the mutants than the WT except E-19 generated from 0.5% EMS. Chlorophyll 'a' and 'b' in the mutagenic population unveiled a general decrease and was significantly lowest in E-14 (0.2% EMS). A general decrease in the photosynthetic pigments, chlorophyll 'a', total chlorophyll, and chlorophyll *a:b* in the EMS induced mutants, particularly at higher dosimetry may be due to the damage in the thylakoid membrane the buds have undergone during their treatment with the alkylating agent. The damage in the chloroplast might have inhibited the catabolic and enzymatic activity. Wani *et al.* (2011) observed an increase in the frequency of chlorophyll mutation at higher mutagen dose. Alterations in the photosynthetic pigment of EMS induced mutants have also been reported in banana by Bidabadi *et al.* (2011) and in cowpea by Gnanamurthy and Dhanavel (2014). Lowest chlorophyll content in *Abelmoschus esculentus* treated with 3% EMS has been reported by Deepthi and Ramesh (2016).

Biochemical parameters

Total phenol: The total phenol content in the EMS derived mutants as adjudged against the WT exhibited an ascending increase in the TPC values and was maximum in mutants created from 0.5% EMS (Table 2). Increase in the phenol content at higher mutagen dose showed a positive correlation with the antioxidant enzymes and could be aptly correlated with plant resistance mechanism for overcoming stress. Ghani *et al.* (2014) found a significant increase in total phenol concentrations in *in-vitro* raised plants of *Gerbera jamesonii* with increasing dose of EMS treatment exhibiting maximum values at the highest dose of 1% EMS. Similarly, Rime *et al.* (2019) reported an increase in the total phenol content in mango leaf in 0.8% of EMS treated mutants.

Proline: A trend analogous to phenol content was also witnessed in the proline content, thus exhibiting a correlated

Table 1 Variation in leaf sclerophyll characteristics and photosynthetic pigments of putative Kinnow mutants

Code of mutant	Leaf area (cm ²)	Leaf fresh weight (g)	Leaf dry weight (g)	Specific leaf area (cm ² /g)	Specific leaf weight (g/cm ²)	Density of foliage tissue (g/kg)	Succulence (mg H ₂ O/cm ²)	Chlorophyll <i>a</i> (mg/g FW)	Chlorophyll <i>b</i> (mg/g FW)	Total chlorophyll (mg/g FW)	Chlorophyll <i>a:b</i>
WT	168.65 ^c	4.49 ^e	1.77 ^{de}	95.56 ^e	0.0105 ^h	392.88 ^{fe}	0.0162 ^{hi}	1.66 ^a	0.31 ^{ki}	1.97 ^b	5.39 ^a
E-1	209.86 ^a	6.60 ^a	1.89 ^c	111.05 ^a	0.0090 ^k	286.57 ^{ij}	0.0224 ^e	1.67 ^a	0.53 ^{bc}	2.19 ^a	3.17 ^{fedg}
E-2	184.79 ^b	5.89 ^b	1.81 ^d	102.09 ^c	0.0098 ^{ij}	307.18 ^h	0.0221 ^e	1.48 ^b	0.52 ^{bcd}	2.00 ^b	2.88 ^{ihg}
E-3	156.65 ^{ed}	4.18 ^g	1.52 ^g	103.17 ^{cb}	0.0097 ^j	363.75 ^g	0.0170 ^{hg}	1.45 ^{cb}	0.40 ^{hg}	1.85 ^{de}	3.66 ^{cbd}
E-4	157.53 ^d	4.32 ^f	1.82 ^{de}	86.43 ^{jih}	0.0116 ^{edf}	421.95 ^{bc}	0.0159 ^{ij}	1.44 ^{cbd}	0.43 ^{efg}	1.88 ^c	3.33 ^{feedg}
E-5	165.35 ^c	4.52 ^e	1.98 ^b	83.52 ^j	0.0120 ^d	438.28 ^a	0.0153 ^{ij}	1.44 ^{cbd}	0.38 ^{hig}	1.82 ^{dee}	3.83 ^b
E-6	143.73 ^{hg}	4.15 ^g	1.72 ^e	83.83 ^{ji}	0.0120 ^d	413.44 ^{cd}	0.0170 ^{hg}	1.36 ^e	0.42 ^{efg}	1.78 ^{dfe}	3.23 ^{feedg}
E-7	149.63 ^f	5.33 ^c	1.53 ^g	98.17 ^{ed}	0.0102 ^{ih}	286.47 ^{ij}	0.0254 ^d	1.39 ^{ed}	0.46 ^{efd}	1.85 ^{de}	3.03 ^{ihg}
E-8	145.24 ^g	5.27 ^c	1.37 ^{ih}	105.86 ^b	0.0095 ^{kj}	260.29 ^l	0.0269 ^c	1.41 ^{ced}	0.42 ^{hig}	1.83 ^{dee}	3.36 ^{feedbg}
E-9	142.80 ^{hg}	3.84 ^h	1.62 ^f	88.43 ^{gh}	0.0113 ^{gf}	420.50 ^{bcd}	0.0156 ^{ij}	1.42 ^{cd}	0.55 ^{ba}	1.97 ^b	2.61 ^{ih}
E-10	153.80 ^e	4.68 ^d	2.07 ^a	74.55 ^{lk}	0.0134 ^{cb}	441.33 ^a	0.0170 ^{hg}	1.26 ^f	0.48 ^{ecd}	1.74 ^f	2.66 ^{ih}
E-11	140.68 ^h	4.73 ^d	2.06 ^a	68.30 ^m	0.0147 ^a	435.71 ^{ba}	0.0190 ^f	1.26 ^f	0.50 ^{bed}	1.76 ^{fe}	2.56 ^{ji}
E-12	112.94 ^k	3.52 ⁱ	1.56 ^{gf}	72.65 ^l	0.0138 ^b	441.42 ^a	0.0174 ^g	1.13 ^g	0.37 ^{hig}	1.50 ^g	3.08 ^{fhg}
E-13	117.58 ^j	3.31 ^j	1.40 ^h	84.40 ^{ji}	0.0119 ^{ed}	421.21 ^{bc}	0.0163 ^{hi}	1.15 ^g	0.32 ^{kj}	1.47 ^{hgi}	3.67 ^{cb}
E-14	132.78 ⁱ	4.74 ^d	1.31 ^{ij}	101.29 ^{cd}	0.0099 ^{ij}	276.99 ^{kj}	0.0258 ^d	1.23 ^f	0.59 ^a	1.82 ^{dfee}	2.09 ^j
E-15	110.70 ^k	4.40 ^{fe}	1.30 ^j	85.38 ^{jih}	0.0117 ^{edf}	294.98 ^{ij}	0.0280 ^b	1.16 ^g	0.32 ^{kij}	1.48 ^{hg}	3.62 ^{cebd}
E-16	101.65 ^l	4.17 ^g	1.13 ^k	90.22 ^{gf}	0.0111 ^g	270.20 ^{kl}	0.0300 ^a	1.11 ^{hg}	0.31 ^k	1.42 ^{hi}	3.69 ^{cb}
E-17	82.30 ⁿ	2.65 ^l	0.81 ^m	102.26 ^{cb}	0.0098 ^{ij}	304.02 ^h	0.0224 ^e	1.07 ^{hi}	0.34 ^{kij}	1.40 ^{hi}	3.16 ^{feg}
E-18	103.93 ^l	2.81 ^k	1.14 ^k	91.35 ^f	0.0110 ^g	405.01 ^{ed}	0.0161 ^{hij}	1.06 ^{hi}	0.33 ^{kij}	1.39 ^{ji}	3.31 ^{feedg}
E-19	71.61 ^o	2.49 ^m	0.94 ^l	76.30 ^k	0.0131 ^c	379.41 ^{fg}	0.0216 ^e	1.02 ⁱ	0.29 ^k	1.31 ^j	3.55 ^{feedbd}
E-20	93.96 ^m	2.50 ^m	1.08 ^k	87.37 ^{gh}	0.0115 ^{egf}	431.46 ^{ba}	0.0151 ^j	1.11 ^{hg}	0.34 ^{kij}	1.44 ^{hgi}	3.33 ^{feedg}
LSD (P ≤ 0.05)	3.71	0.13	0.07	3.63	0.0005	15.84	0.0010	0.05	0.05	0.08	0.49

Table 2 Variation in biochemical parameters and antioxidants enzymes activity of putative Kinnow mutants

Code of mutant	Total phenol (mg/g FW)	Proline (μ M/g FW)	Total soluble protein (mg/g FW)	Superoxide dismutase (mg/ protein min)	Catalase (μ mol of H_2O_2 hydrolysed mg/protein min)	Peroxidase (μ mol tetra-guaiacol formed mg/ protein min)	Glutathione reductase (mg/ protein min)
WT	48.79 ^h	0.36 ^h	9.11 ^j	0.92 ^f	24.39 ^h	1.30 ^{dfge}	1.15 ^c
E-1	58.13 ^{gf}	0.71 ^g	13.74 ^h	1.09 ^e	24.93 ^h	1.22 ^{hfge}	0.82 ^d
E-2	58.27 ^{gf}	0.72 ^g	14.99 ^{fe}	1.08 ^e	24.12 ^h	1.15 ^{hi}	0.76 ^d
E-3	57.94 ^g	0.78 ^{ef}	16.35 ^{dc}	1.09 ^e	24.47 ^h	1.18 ^{hgi}	0.74 ^d
E-4	57.94 ^g	0.74 ^{gf}	14.89 ^{fe}	1.01 ^{fe}	24.28 ^h	1.20 ^{hfg}	0.81 ^d
E-5	59.41 ^f	0.74 ^{gf}	14.92 ^{fe}	1.06 ^e	24.67 ^h	1.03 ⁱ	0.81 ^d
E-6	64.04 ^{ed}	0.84 ^d	14.75 ^{fe}	1.58 ^{cd}	28.35 ^g	1.15 ^{hi}	0.88 ^d
E-7	62.87 ^e	0.80 ^{ed}	16.18 ^d	1.52 ^d	29.01 ^{gf}	1.14 ^{hi}	0.90 ^d
E-8	64.74 ^d	0.81 ^{ed}	17.14 ^{ba}	1.50 ^d	28.87 ^{gf}	1.13 ^{hi}	0.89 ^d
E-9	64.08 ^{ed}	0.82 ^d	17.38 ^{ba}	1.53 ^d	29.27 ^{gf}	1.20 ^{hfg}	0.83 ^d
E-10	64.38 ^d	0.83 ^d	14.53 ^{fg}	1.51 ^d	29.70 ^{egf}	1.20 ^{hfg}	0.83 ^d
E-11	68.68 ^c	0.90 ^c	14.96 ^{fe}	1.69 ^{eb}	31.39 ^{ed}	1.34 ^{dfce}	1.08 ^c
E-12	69.71 ^c	0.94 ^{bac}	15.24 ^e	1.71 ^b	31.63 ^d	1.37 ^{dce}	1.21 ^{cb}
E-13	68.79 ^c	0.95 ^{ba}	15.23 ^e	1.72 ^b	31.26 ^{ed}	1.49 ^c	1.22 ^{cb}
E-14	69.12 ^c	0.94 ^{bac}	17.75 ^a	1.69 ^{eb}	30.05 ^{egdf}	1.40 ^{dc}	1.22 ^{cb}
E-15	69.12 ^c	0.97 ^a	12.76 ⁱ	1.78 ^b	30.51 ^{edf}	1.44 ^{dc}	1.37 ^b
E-16	82.06 ^{ba}	0.92 ^{bc}	13.82 ^h	2.00 ^a	41.79 ^{ba}	1.95 ^{ba}	1.75 ^a
E-17	81.47 ^{ba}	0.84 ^d	13.95 ^{hg}	2.01 ^a	42.32 ^a	2.00 ^a	1.73 ^a
E-18	80.85 ^b	0.83 ^d	16.90 ^{bc}	2.00 ^a	39.79 ^c	1.95 ^{ba}	1.81 ^a
E-19	82.43 ^a	0.83 ^d	14.48 ^{fg}	2.10 ^a	40.34 ^{bc}	1.84 ^b	1.89 ^a
E-20	81.99 ^{ba}	0.81 ^{ed}	16.20 ^d	2.06 ^a	42.32 ^a	1.81 ^b	1.89 ^a
LSD (P $\leq$ 0.05)	1.46	0.04	0.64	0.12	1.88	0.15	0.17

dose-dependent increase of more than twofold in the mutants E-13 and E-15 derived from 0.2% EMS (Table 2). The increase in proline content in the mutants as compared to WT confirms osmo tolerance in the mutagenic population. Such findings have also been supported by Mallick *et al.* (2016) in Kinnow mandarin and Kumar and Pandey (2019) in *Coriandrum sativum*.

Total soluble protein: The TSP content in EMS induced mutants exhibited a trend similar to the proline content, thereby revealing an increase of almost two-fold in the mutants E-14 (0.2% EMS), E-8 and E-9 (0.1% EMS) (Table 2). Higher TSP in these mutants might have accumulated under stress due to the alterations in the protein pathway. Very little effort has been made to understand the effect of an alkylating agent such as EMS on the leaf protein alteration in fruit crops. The present finding is in agreement with Kumar and Munirajappa (2013) who reported higher protein accumulation in mulberry leaves generated from 0.35% EMS. Stimulated increase in total protein concentrations at 1% EMS have also been reported by Ghani *et al.* (2014) in gerbera (*Gerbera jamesonii* Hook.).

Antioxidant enzymes activity: Anti-oxidant systems provide a protective mechanism to eliminate or reduce ROS levels in plant cells (Kumari *et al.* 2010). The activity of

antioxidant enzymes in contrast to WT demonstrated a dose-dependent increase in SOD, CAT, POX and GR activity and was significantly up-regulated in the mutants E-16 to E-20 induced from 0.5% EMS (Table 2). As adjudged against the WT, decrease in the GR activity was noted in the mutants E-1 to E-10 developed from 0.05-0.1% EMS. The increase in the antioxidant enzyme at higher dosimetry in the putative mutants may be ascribed to its role in protecting the plant cells from the destructive effects of ROS as key components of the cellular antioxidant defense system. The finding of the present study is in consonance with Acedo (1983) and Rime *et al.* (2019) who reported elevated antioxidant enzymes in the EMS induced mutants of *Arabidopsis* and mango.

This study showed that chemical mutagens have significant effects on the physiology of Kinnow plants. The decreases observed in chlorophyll concentrations confirmed the negative effects of EMS on plant pigment levels. The detection of higher levels of total proteins and phenolic compounds in all mutants supports the roles played by these molecules in the plant defence system. Mutagenesis also led to the activation of enzymatic antioxidant defence system by increasing its capacity to scavenge ROS. Thus, anti-oxidant enzyme activities can be used as indicators of the sensitivity of plants to EMS and developing genotypes

for use in the future breeding programme for developing varieties of traits of interest.

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