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Hormonal changes in gamma irradiated and EMS induced mutants of Kinnow mandarin (*Citrus nobilis* Loureiro × *Citrus deliciosa* Tenora)

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Abstract
The study was performed on the selected Kinnow mandarin mutants developed through varying doses of gamma irradiation *viz*., 15, 20, 25 and 30 Gy and ethyl methanesulfonate (EMS) (0.05%, 0.1%, 0.2% and 0.5%). From the varied population, five putative mutants from each treatment were selected to study the phytohormone content and compared with the wild type (WT). The mutants showed substantial variability for hormone content as compared to WT. Irradiation treatment enhanced IAA content by 188.78-247.23% in the mutants G_7-G_9, while 79.57-93.46% enhancement in ABA content was assayed in the mutants G_6 and G_7 developed at 20 Gy. The EMS treatment also increased the IAA content in the mutant populations derived from higher doses and the maximum (99.91 ng g^{-1} FW) was recorded in E_{18} followed by E_{19} (96.68 ng The ABA levels were found to be elevated in all EMS induced mutants which was highest in E_{18} (381.65 ng g^{-1} FW) followed by E_{20} (345.75 ng g^{-1} FW).

Keywords: Kinnow mandarin, gamma irradiation, EMS, IAA, ABA

Introduction
Kinnow mandarin (*Citrus nobilis* Loureiro x *Citrus deliciosa* Tenora) has emerged as a predominant mandarin and is widely grown in the arid, semi-arid and lower foot hills of the Indian subcontinent. It is the most favoured mandarin from the consumers and grower's point of view because of its attractive appearance, higher juice recovery, extended market availability, better yield and high economic returns (Kumar *et al*., 2018) [6]. All these attributes have enabled this mandarin to occupy a place of eminence owing to its major share in the area and production of citrus grown in India (Kumar *et al*., 2019) [5]. Despite the various positive traits, vigorous plant growth, alternate bearing, susceptibility to salinity and *Phytophthora* and presence of the higher number of seeds in fruits limits its productivity and scope in the processing sector (Mallick *et al*., 2016) [8]. Thus, seedlessness, small tree size, regular bearing, tolerant/resistant plant to various biotic and abiotic stresses are some of the main objectives of Kinnow breeding programs which however, are difficult to achieve through conventional breeding because of their high hetrozygosity, quantitative inheritance and long juvenile phase.
Mutagenesis on the other hand has the potential for creating variability by changing one or a few trait specific genes that can contribute to fruit improvement (Predieri, 2001) [10]. The variability normally observed in the mutated plants is in the form of physiological and biochemical alterations which result due to disturbances in protein synthesis, hormonal imbalance, leaf and water gas-exchange capacity**,** enzyme activity etc., depending on the engrossed irradiation dose (Xiuzhen, 1994; Kiong *et al*., 2008; Wi *et al*., 2006) [17, 4, 16].
Phytohormones play a key role in regulating developmental processes and growth, signalling networks and most physiological functions within plants including shoot divaricating/branching, adjustment of fruit set and development, another development, and responses to biotic and abiotic stress (Quecini *et al*., 2007; Santner *et al*., 2009) [12, 14]. Elevated levels of endogenous phytohormones have been observed during parthenocarpic fruit set (George *et al*., 1984; Talon *et al*., 1990) [3, 15], suggesting that the increased supply of phytohormones to fruit from sources other than seeds may be sufficient to induce fruit growth. Therefore, the present study was aimed to analyse the phytohormone changes in induced mutants of Kinnow mandarin.

Materials and Methods

Plant materials and growing conditions

Kinnow mutants were developed through the treatment of different doses of gamma irradiation (15, 20, 25 and 30 Gray) and ethyl methanesulphonate (EMS) (0.05%, 0.1%, 0.2% and 0.5%) (Table 1). These mutants and wild type were developed from the same source of bud wood, budded on *Jatti Khatti* (*Citrus jambhiri* Lush) rootstock, and field planted during 2011 at 3 m × 3 m at the Division of Fruits and Horticultural Technology, ICAR-IARI, New Delhi. The experimental site (77° 12' E; 28° 40' N 228.61 masl) is characterized as semiarid and subtropical, with hot and dry summers and cold winter. The mean annual rainfall is 710 mm of which more than 75 per cent is received during monsoon season (July to September). The soil type was sandy loam with bulk density 1.58 g cm^{-3}, pH of 7.4, electrical conductivity [EC; 1:2 (W/V) in water] of 0.34 dSm^{-1}, organic carbon content of 0.39% (w/w) and a soil N, P and K concentration of 159.23, 536.1 and 314.78 kg ha^{-1} respectively. The mean available Fe, Mn, Cu and Zn concentration in the soil was 9.35, 22.67, 7.52 and 4.83 mg kg^{-1} soil respectively. Irrigation was applied through drip system from mid-January to June and from October to December, with water having an EC of 1.3 dSm^{-1}. The nutrition (Farmyard manure 25 kg, 600 g N, 400 g P and 600 g K per tree/year) were applied in two split doses *viz*., February and September. Rest of the management practices were followed as per the accordance of strict schedule of cultural operations.

Table 1: Description of Kinnow mutants developed through gamma irradiation and EMS

Gamma irradiation dose	Mutant population	Code of mutant	EMS concentration	Mutant population	Code of mutant
Non treated	Wild type	WT	Non treated	Wild type	WT
15 Gray	15-1	G_1	0.05%	0.05-1	E_1
15 Gray	15-2	G_2	0.05%	0.05-2	E_2
15 Gray	15-3	G_3	0.05%	0.05-3	E_3
15 Gray	15-4	G_4	0.05%	0.05-4	E_4
15 Gray	15-5	G_5	0.05%	0.05-5	E_5
20 Gray	20-1	G_6	0.1%	0.1-1	E_6
20 Gray	20-2	G_7	0.1%	0.1-2	E_7
20 Gray	20-3	G_8	0.1%	0.1-3	E_8
20 Gray	20-4	G_9	0.1%	0.1-4	E_9
20 Gray	20-5	G_{10}	0.1%	0.1-5	E_{10}
25 Gray	25-1	G_{11}	0.2%	0.2-1	E_{11}
25 Gray	25-2	G_{12}	0.2%	0.2-2	E_{12}
25 Gray	25-3	G_{13}	0.2%	0.2-3	E_{13}
25 Gray	25-4	G_{14}	0.2%	0.2-4	E_{14}
25 Gray	25-5	G_{15}	0.2%	0.2-5	E_{15}
30 Gray	30-1	G_{16}	0.5%	0.5-1	E_{16}
30 Gray	30-2	G_{17}	0.5%	0.5-2	E_{17}
30 Gray	30-3	G_{18}	0.5%	0.5-3	E_{18}
30 Gray	30-4	G_{19}	0.5%	0.5-4	E_{19}
30 Gray	30-5	G_{20}	0.5%	0.5-5	E_{20}

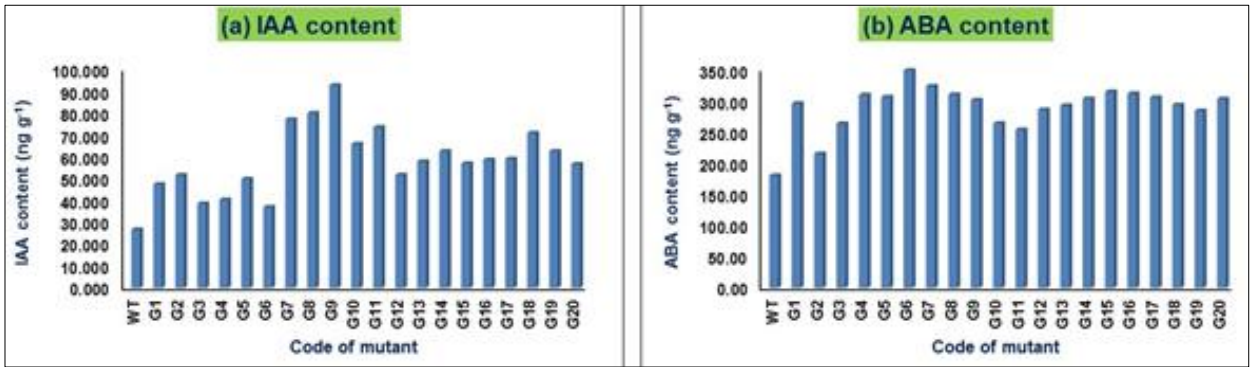

Fig 1: Changes in endogenous (a) IAA and (b) ABA contents in gamma irradiated induced putative Kinnow mutants

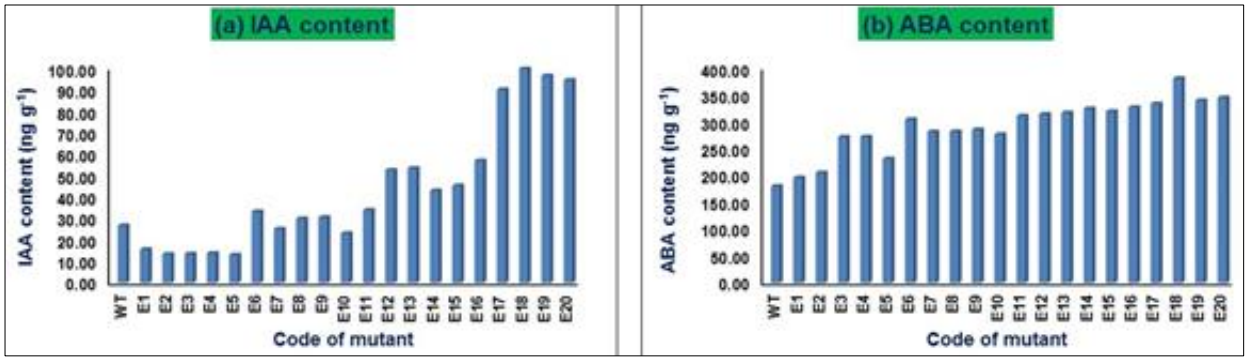

Fig 2: Changes in endogenous (a) IAA and (b) ABA contents in EMS derived putative Kinnow mutants

Phytohormones

Extraction of indole-3-acetic acid (IAA)

The fresh leaf (1.0 g) was used for extraction of endogenous IAA (Fu *et al*., 2011) [2] with minor modifications in extraction (repeated overnight extraction of IAA from samples with methanol) and final sample volume for IAA reconstitution after vacuum evaporation (300 µL methanol). Samples were ground to a fine powder in a mortar and pestle with liquid nitrogen and extracted in methanol (2.5 mL g^{-1} fresh weight). The extract was centrifuged at 15,000× g for 10 min at 4°C (model-HERMLE Z 323K). The resulting supernatant was transferred to a new tube and concentrated using a vacuum concentrator (Speed Vac, Thermo Scientific) until the volume decreased to less than one-tenth of the initial volume. One volume of water was added in the sample before partitioning with ethyl acetate. The pH of the solution was adjusted 9 -10 with 1 M KOH to keep IAA ionized and then partitioned with 100% ethyl acetate. The aqueous and organic phases were separated by centrifugation (15,000× g for 2 min), and the lower aqueous phase was transferred to a new tube. The pH of the solution was lowered to below 3 with acetic acid to conserve IAA in protonated form. The acidic sample was partitioned again with ethyl acetate and cleared by centrifugation. The upper organic phase was recovered and completely dried in a vacuum concentrator and dissolved in 300 µL of methanol thereafter. Before injecting the sample into HPLC, the samples were filtered with 0.45 µm PVDF membrane micro syringe filter. The samples were then injected (20 µL) and analysed by HPLC.

HPLC conditions

Chromatographic separation was performed on an Agilent 1200 series HPLC system (Agilent Technologies Inc., USA), which included a degasser, a quaternary pump, an auto-sampler and FLD. A Zorbax Eclipse XDB-C18 reversed-phase column (5 µm, 4.6 × 250 mm, Agilent) at the temperature of 30°C was used as the separation platform for IAA. Method control, data acquisition, and analysis were done through the Chem Station. An injection volume of 20 µL was used for each analysis.

Solvent system and detector

The mobile phase included 90 per cent methanol with 0.3 per cent acetic acid (solvent A) and 10 per cent methanol with 0.3 per cent acetic acid (solvent B). A post time of 5 min was allowed after the gradient programme. The FLD was set with excitation wave length at 280 nm and emission wave length at 360 nm.

Extraction of abscisic acid (ABA)

Abscisic acid in the leaf sample was estimated through HPLC, using the method suggested by Zeevaart (1980) [18] with minor modifications. Collected leaf samples (1g) was frozen in liquid nitrogen, pulverized and stored at -20 oC until further use. Frozen samples were ground into a fine powder using mortar and pestle in liquid nitrogen, and extracted thrice with 10 ml of 80% v/v acetone (80 ml acetone, one ml glacial acetic acid and 100 mg of 2, 6 di-tert-butyl 4-methyl phenol in a total volume of 100 mL). The extract was filtered through Whatman No. 1 filter, and The filtrate was transferred to the boiling flask of rotary flash vacuum evaporator for removing the acetone. As the acetone was evaporated, the lipid soluble material was deposited on the walls of the boiling flask (round bottomed). This was dissolved in 1ml of 1% acetic acid solution, and the amber coloured aqueous solution was transferred into small vials (1ml). Before injecting the sample into HPLC, the samples were filtered with 0.45 µm PVDF membrane micro syringe filter.

HPLC conditions

The preparative HPLC system was the same as that used for IAA extraction.

Solvent system and detector

The separation was carried out on ZORBAX Eclipse XDB-C18 column (250x4.6mm, 5 µm) at 30ºC with mobile phase composed of 1% acetic acid in 95% methanol in isocratic mode at a flow rate of 1mL min^{-1}. The detection was monitored at variable wavelength detector at 265 nm.

Results

The overall trend of IAA concentration in the gamma irradiated mutants as compared to WT (26.76 ng/g) exhibited significantly higher peak values in the mutant G_9 (92.92 ng/g), G_8 (80.18 ng/g) and G_7 (77.28 ng/g) exposed through the radiation of 20 Gy (Fig. 1a). It was also observed that gamma irradiation mutagenesis notably induced ABA accumulation in the mutants. ABA concentration peaked in the mutants G_6 (349 ng/g) and G_7 (323.94 ng/g) developed at 20 Gy. Among the gamma irradiated mutant population, relatively lower ABA was noticed in the mutant G_2 (215.36 ng/g) although, it was higher than the values attained in the WT (180.39 ng/g) (Fig. 1b).

The treatment of EMS with different concentrations also significantly increased the IAA content in the mutant populations derived from higher doses. The IAA content in EMS derived mutants was statistically different in all the treatments as compared to WT (26.76 ng g^{-1} FW) and the maximum (99.91 ng g^{-1} FW) was recorded in E_{18} followed by E_{19} (96.68 ng g^{-1} FW) and E_{20} (94.65 ng g^{-1} FW). The minimum value (13.05 ng g^{-1} FW) was recorded in E_5 which was not statistically different from E_2 (13.39 ng g^{-1} FW), E_3 (13.53 ng g^{-1} FW) and E_4 (13.85 ng g^{-1} FW) (Fig. 2a). The ABA levels were found to be elevated in all mutants and exhibited significant variation among the mutants. The ABA level was highest in E_{18} (381.65 ng g^{-1} FW) followed by E_{20} (345.75 ng g^{-1} FW) which was not statistically different from E_{19} (340.29 ng g^{-1} FW) and E_{17} (334.32 ng g^{-1} FW) and lowest in WT (180.39 ng g^{-1} FW) (Fig. 2b).

Discussion

In *Citrus* species, several growth and developmental processes are determined by phytohormones (Quecini *et al*., 2007) [12]. In our study, fluctuating trends were observed for the phytohormnes studied. However, both IAA and ABA peak values were assayed in the mutants developed at 20 Gy suggesting its adaptive response to stress caused by irradiation. Bhatt *et al*. (2008) [1] suggested that there might be two reasons of radiation effect on increasing endogenous hormones: Irradiation causes *de novo* synthesis of free hormone level to overcome the physical stress, or due to radiation effect, conjugated forms are converted to free form. The findings are in line with Latif *et al*. (2011) [7] and Qi *et al*. (2015) [11], who reported that low doses of gamma irradiation stimulated more synthesis whereas high doses inhibited phytohormones. Similarly, In EMS derived mutants both IAA and ABA content increased in all the mutants. It was found to be more in less vigour mutants developed at higher concentration, so plant height can be negatively correlated with ABA content. The findings are in accordance with Murti

& Upreti, 2003 and Rime *et al*., 2019 [9, 13], who reported elevated ABA level in less vigorous plants of mango.

Conclusion

From the present experimental findings, it can be concluded that different doses of gamma irradiation and EMS changed the hormonal profiling of mutants as compared to wild type.

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Conflict of interest

Authors declare no conflict of interest for this work.

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