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Irradiation effect on leaf sclerophylly, gas exchange and stomata in sweet orange (*Citrus sinensis*)

KULDEEP SINGH[1], O P AWASTHI[1]*, AWTAR SINGH[1], REENA PRUSTY[1] and PRACHI YADAV[1]

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

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ABSTRACT

The study was carried out in mutated population of sweet orange [*Citrus sinensis* (L.) Osbeck] cv. Mosambi developed from varying dose of gamma rays (10-35 Gy) to determine its effect on leaf sclerophylly, photosynthesis and leaf anatomical characters at the Division of Fruits and Horticultural Technology, ICAR-Indian Agricultural Research Institute, New Delhi during 2017 and 2018. As compared to wild type leaf area, fresh and dry mass was stimulated by 19.53, 18.42 and 20.37% in mutant GS-3 developed from 10 Gy, while in the mutants GS-33 and GS-35 developed from 35 Gy >50% inhibitory effects were noticed. Leaves were however, 82.06% more succulent in the mutant developed from 30 Gy. Mutated population showed dose dependent effect with regard to SLA, SLW and DFT. A significant decline in the photosynthetic rate and stomatal conductance was noticed at 35 Gy in GS-32, whereas mutant GS-34 developed from the same irradiation dose had the minimum transpiration rate. Stomata length and width in the mutants GS-17 and GS-15 as compared to wild type registered an increase of 25.50% and 15.96%. A general reduction in stomata number was observed in the mutated populations. Our result indicated alteration in traits of the mutated population which are of significance and can be used as a selection criterion for improvement of sweet orange.

Keywords: Leaf sclerophylly, Leaf gas exchange, Mutant, Stomata, Sweet orange

Citrus is amongst the top ranked fruits of the world and is the third most important fruit of India. Amongst the citrus fruits grown in India, sweet orange [*Citrus sinensis* (L.) Osbeck)] occupies second position after mandarin and is grown in an area of 0.18 million ha with an estimated production of 2.87 million tonnes and contributes about 23.47% of total citrus production in the country (Anonymous 2018). The fruits are the prime source of vitamin C and so immense are its nutritional value that it has also been recommended as immunity booster against Covid-19. Consumers preference of sweet orange is normally in the form of juice which however, during the process of juice extraction; the seeds get crushed resulting in increased limonene content and bitterness of the juice. Despite the several available cultivars in sweet orange, still there is lack of varieties which possess all the desirable traits and the most important being the availability of low seeded varieties. Conventional breeding although have led to the development of certain promising varieties in other fruit crops, the progress in citrus had rather been slow because of its complicated genetic system. To overcome the limitations of traditional breeding, mutagenesis as a tool have played a significant role in the development of several classical mutants in fruit crops including citrus having desirable traits such as seedlessness, dwarfness, tolerance to various biotic and abiotic stresses etc. (Ahloowalia 2001). These traits are achieved due to the modifications in morphological and physiological alterations in plants which provide the basic variability for plant improvement (Broertjes and Van Harten 2013, Mallick *et al.* 2016). In the present study, an attempt has been made to develop a genetic variation in sweet orange through different doses of gamma rays with an objective to analyse the genetic variability in the selected pre-bearing putative mutants which could be correlated at the later stages for traits of genetic interest such as low seediness or tolerance to the various stresses.

MATERIALS AND METHODS

The experiment was conducted on two years old mutagenic population of sweet orange cv. Mosambi developed with different dosages of gamma rays, viz. 10, 15, 20, 25, 30 and 35 gray. The mutants were established *in-situ* on *Jatti Khatti* rootstock at spacing of 3 m × 3 m and maintained at the experimental orchard of Fruits and Horticultural Technology, ICAR-IARI, New Delhi following the normal recommended package of practices. From the diverse population, six mutants from each treatment and four mutants from 35 Gy which showed deviation in morpho-functional traits as compared to wild type were investigated

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

for the variations in leaf characters, gas exchange parameters and cellular behaviour for two consecutive years 2017 and 2018.The mutants were code GS-0 (wild type/control), GS-1 to GS-6 (10 Gy), GS-7 to GS-12 (15 Gy), GS-13 to GS-18 (20 Gy), GS-19 to GS-24 (25 Gy), GS-25 to GS-30 (30 Gy) and GS-31 to GS-34 (35 Gy).

To examine the alterations in leaf sclerophylly, 10 mature, two and half-month-old leaf of the rainy season flush were collected from different directions (N, S, E and W) as replicates during October. The collected leaves were brought to the laboratory in ice box and weighed to determine their fresh mass (FM). Thereafter, leaf area (LA) was measured using a Li-Cor, LI 3100 area meter (Li-Cor, USA). The leaves were then oven-dried (70°C ± 1°C) for 48 h and their dry mass (DM) was determined after the samples attained a constant weight. The formulae suggested by Ennajeh *et al.* (2010) was used for determining the indices of leaf physiological parameters which included specific leaf area (SLA= LA/ DM: in cm^2/g DW), specific leaf weight (SLW = DW/LA: in g/cm^2 LA), density of foliar tissue (D = DW/FW X 1000: in g/kg) and succulency [S = (FW-DW)/LA: in mg H_2O/cm^2]. Net Photosynthesis (*A*), Stomatal Conductance (*gs*), Intercellular CO_2 concentration (*Ci*) and transpiration (*E*) were recorded on 60-70 day fully mature leaf (4^{th} leaf from tip of shoot) of the spring season flush in cardinal directions of the compass. The observations were recorded using LCi-SD Ultra Compact Photosynthesis System between 11.00 and 12.00 h at light saturating intensity. Averages of 12 readings/treatment were recorded.

Leaf ordained for studying the anatomical characteristics was performed on fully mature expanded leaf by preparing impressions of the abaxial surfaces as described by Sampson (1961). Four leaves per treatment and four fields per leaf were taken for recording the observations for stomatal density, length and width. Stomatal density was assessed by counting the number of stomata per field (1000 μm^2) of view at 40x magnification using Motic BA400 microscope with inbuilt digital camera. Stomata length and width were measured using motic software. Mean replicated data of years were statistically analysed in completely randomized block design (CRBD) with 4 replications to measure standard error, subjected to analysis of variance (ANOVA) using SAS package (9.3 SAS Institute, Inc., USA) followed by Tukey's Honest test. P values ≤0.05 were considered as significant. Relationships among leaf sclerophylly and stomatal parameters were computed using Pearson's simple correlation by SPSS software.

RESULTS AND DISCUSSION

Leaf sclerophylly measured across the unirradiated (wild type) and irradiated treatments exhibited significant changes in the leaf characteristics (Table 1). Mean leaf area, leaf fresh weight and leaf dry weight as compared to the wild type increased by 19.53, 18.42 and 20.37% respectively in the mutant GS-3 developed from the lowest irradiation dose of 10 Gy, while a significant decrease of more than 50% in the related parameters were recorded in the mutants GS-33 and GS-35 developed with highest 35 Gy gamma dose (Table 1). Reverse trend was however, recorded with respect to leaf succulency, thus exhibiting an increase of 82.06% and a decrease of 25.95% in the mutants GS-30 and GS-8 developed from 30 and 15 Gy, respectively. Mutants developed from the different doses of gamma rays showed dose dependent effect with regard to SLA, SLW and DFT (Table 1). Irrespective of the mutants developed from varying doses of gamma rays, a general reduction in SLA was noticed, except mutants GS-4 and GS-2 which recorded an increase of 27.08 and 6.38% developed from 10 Gy. In contrast, a general increase in SLW and DFT were recorded in all the mutant population over wild type, except GS-2 and GS-4 which registered a varying decrease of 6.91 and 19.74% in SLW, whereas DFT in the said mutants recorded a decrease of 3.02 and 8.4%.

Ionizing radiation may have positive, negative or null effects on plant morphology depending on the irradiation intensity (De Micco *et al.* 2011). Decrease in leaf area, fresh and dry weight in the mutant at the lowest dosimetery may be attributed to induced hormesis effect. Similar low dose effect of gamma ray effect has been reported in sweet orange and grape fruit by Hearne (1984). A deleterious effect in leaf area, fresh and dry weight at higher dosimetery indicates changes arising due to physiological and metabolic disturbances such as malfunctioning of various phyto-hormones which causes ultimate changes in chemical patterns leading to morphological variation (Sharma *et al.* 2013). Modification in leaf succulency and Density of Foliar Tissue (DFT) at higher and lower irradiation doses as observed in our study is in consistent with the findings of Mallick *et al.* (2016) who reported more succulent leaves with significant reduction in leaf area and density of foliar tissue at higher irradiation doses.

Microscopy observations of leaf surface imprints in wild type and mutated plants are presented in Table 1. In contrast to wild type, a striking increase of 25.50% and 15.96% in stomata length and width was recorded in the mutants GS-17 and GS-15 developed from 20 Gy, whereas a decrease of 16.35% and 22.69% was noticed in the mutant GS-10 developed from 15 Gy. Prominent decrease in stomata number as compared to wild type was recorded in the mutated population and stomata number was envisaged minimum in mutant GS-4 which had a statistical parity with mutants GS-12, GS-13, GS-15, GS-16, GS-32 and GS-33. Observation of leaf abaxial surface and analysis of variance showed that there were anatomical alterations in the kinnow mutants. Transitional doses of gamma rays showed higher values of stomata length and width, while a lower value compared to wild type was observed in a step down irradiated mutant. The observation suggests that the intermediary doses were more effective in inducing genetic damage and mutation frequencies than very low or high irradiation dose. Similar anatomical changes at intermediary radiation doses have also been reported by Widiastuti *et al.* (2010) in mangosteen (*Gacinia mangostana*). The radiation effect on reduced number of stomata as observed in our

Table 1 Alteration in leaf sclerophylly and stomata parameters in the gamma irradiated mutants

Treatment	Leaf area (cm^2)	Leaf fresh weight (g)	Leaf dry weight (g)	Succulency (mg H_2O/ cm^2)	Specific leaf area (cm^2/g)	Specific leaf weight (g/cm^2)	Density of foliage tissue (g/kg)	No. of stomata/ 1000 μm^2	Length (µm)	Width (µm)
GS-0	442.55^{b}	15.85^{c}	4.27^{c}	0.0262^{i}	103.58^{c}	0.0097^{v}	269.60^{m}	3.25^{a}	13.45^{fgieh}	$12.03^{eidfhjg}$
GS-1	281.78^{j}	9.43^{q}	2.82^{o}	0.0235^{m}	99.84^{e}	0.0100^{t}	299.30^{k}	2.25^{bc}	14.83^{bdec}	$12.03^{eidfhjg}$
GS-2	339.03^{f}	11.77^{g}	3.08^{m}	0.0256^{kj}	110.19^{b}	0.0091^{w}	261.46^{n}	2.25^{bc}	13.88^{fgdeh}	10.85^{mklj}
GS-3	528.97^{a}	18.77^{a}	5.14^{a}	0.0258^{j}	102.97^{c}	0.0097^{vu}	273.71^{m}	2.50^{bc}	13.75^{fgeh}	12.23^{edfhcg}
GS-4	247.11^{o}	7.60^{w}	1.88^{w}	0.0232^{n}	131.63^{a}	0.0076^{x}	246.95^{o}	2.00^{c}	13.70^{fgeh}	11.80^{eifhjg}
GS-5	342.96^{e}	12.41^{f}	3.38^{j}	0.0264^{i}	101.55^{d}	0.0098^{u}	272.15^{m}	2.25^{bc}	14.43^{fde}	12.55^{ebdfc}
GS-6	289.46^{i}	12.53^{e}	3.93^{e}	0.0297^{d}	73.70^{pq}	0.0136^{fe}	313.38^{j}	2.50^{bc}	13.05^{fgijh}	$12.03^{eidfhjg}$
GS-7	223.43^{u}	8.22^{t}	3.21^{l}	0.0225^{p}	69.67^{t}	0.0144^{a}	390.30^{a}	2.75^{ba}	13.10^{fgijh}	$12.25^{ebdfhcg}$
GS-8	277.59^{k}	8.74^{s}	3.36^{j}	0.0194^{w}	82.60^{ij}	0.0121^{nm}	384.71^{b}	2.75^{ba}	13.78^{fgeh}	$12.35^{ebdfhcg}$
GS-9	246.59^{po}	8.15^{ut}	2.72^{p}	0.0221^{q}	90.82^{f}	0.0110^{s}	333.14^{h}	2.70^{ba}	12.40^{kijh}	11.15^{ikhj}
GS-10	237.17^{r}	7.62^{w}	2.92^{n}	0.0198^{v}	81.22^{kl}	0.0123^{lk}	383.21^{b}	2.70^{ba}	11.25^{k}	9.30^{n}
GS-11	184.15^{z}	7.23^{x}	2.35^{ts}	0.0266^{h}	78.54^{m}	0.0127^{j}	324.38^{i}	2.25^{bc}	14.60^{fbdec}	12.23^{edfhcg}
GS-12	435.05^{c}	16.21^{b}	5.13^{a}	0.0255^{k}	84.77^{h}	0.0118^{p}	316.72^{i}	2.00^{c}	15.40^{bdac}	13.50^{ba}
GS-13	245.01^{p}	10.05^{m}	3.28^{k}	0.0276^{f}	74.70^{po}	0.0134^{fg}	326.37^{i}	2.00^{c}	14.55^{fdec}	12.53^{ebdfc}
GS-14	354.49^{d}	14.31^{d}	4.61^{b}	0.0274^{g}	76.88^{n}	0.0130^{h}	322.22^{i}	2.25^{bc}	14.75^{bdec}	10.95^{iklj}
GS-15	195.85^{x}	6.27^{z}	2.27^{u}	0.0204^{u}	86.20^{g}	0.0116^{q}	362.59^{e}	2.00^{c}	16.18^{ba}	13.95^{a}
GS-16	269.16^{l}	10.97^{i}	3.48^{i}	0.0278^{e}	77.39^{g}	0.0129^{ih}	317.11^{j}	2.00^{c}	14.03^{fgde}	$12.00^{eidfhjg}$
GS-17	319.13^{g}	11.18^{h}	4.07^{d}	0.0223^{p}	78.42^{m}	0.0128^{j}	364.09^{e}	2.25^{bc}	16.88^{a}	11.25^{ikhjg}
GS-18	230.85^{t}	8.10^{u}	3.09^{m}	0.0217^{r}	74.83^{po}	0.0134^{g}	381.09^{cb}	2.25^{bc}	13.73^{fgeh}	11.80^{eifhjg}
GS-19	306.04^{h}	9.86^{n}	3.62^{g}	0.0204^{u}	84.66^{h}	0.0118^{po}	366.54^{e}	2.50^{bc}	14.13^{fgde}	12.83^{ebdac}
GS-20	232.08^{t}	10.39^{k}	3.27^{k}	0.0307^{b}	71.03^{s}	0.0141^{b}	314.45^{j}	2.25^{bc}	12.78^{kgijh}	11.18^{ikhjg}
GS-21	207.24^{w}	7.28^{x}	2.65^{q}	0.0224^{p}	78.28^{m}	0.0128^{ij}	363.66^{e}	2.75^{ba}	13.13^{fgijh}	11.78^{eifhjg}
GS-22	207.39^{w}	8.02^{v}	2.31^{tu}	0.0276^{f}	89.98^{f}	0.0111^{s}	287.27^{l}	2.25^{bc}	14.30^{fgde}	$12.33^{ebdfhcg}$
GS-23	186.75^{y}	7.06^{y}	2.57^{r}	0.0241^{l}	72.67^{rq}	0.0138^{dc}	363.88^{e}	2.25^{bc}	13.45^{fgieh}	$12.15^{eidfhcg}$
GS-24	234.65^{s}	8.02^{v}	3.03^{m}	0.0213^{s}	77.38^{nm}	0.0129^{ih}	378.11^{cd}	2.50^{bc}	14.45^{fde}	12.43^{ebdfcg}
GS-25	280.44^{j}	10.55^{j}	3.83^{f}	0.0240^{l}	73.27^{q}	0.0136^{de}	362.88^{e}	2.50^{bc}	11.30^{k}	10.15^{mkln}
GS-26	245.48^{po}	8.02^{v}	2.81^{o}	0.0212^{s}	87.36^{g}	0.0114^{r}	350.26^{f}	2.75^{ba}	11.65^{kj}	9.68^{mn}
GS-27	265.61^{m}	9.07^{r}	3.53^{hi}	0.0209^{t}	75.20^{o}	0.0133^{g}	389.35^{a}	2.25^{bc}	12.03^{kij}	9.75^{mln}
GS-28	270.17^{l}	9.51^{p}	3.34^{j}	0.0228^{o}	80.91^{kl}	0.0124^{lk}	351.26^{f}	2.25^{bc}	16.10^{bac}	$12.25^{ebdfhcg}$
GS-29	242.83^{q}	10.26^{l}	2.93^{n}	0.0302^{c}	83.03^{i}	0.0120^{n}	285.21^{l}	2.50^{bc}	13.93^{fgdeh}	13.18^{bdac}
GS-30	183.14^{z}	11.12^{h}	2.40^{s}	0.0477^{a}	76.49^{n}	0.0131^{h}	215.31^{p}	2.25^{bc}	14.33^{fgde}	13.38^{bac}
GS-31	258.16^{n}	9.58^{o}	3.59^{hg}	0.0232^{n}	71.98^{rs}	0.0139^{c}	374.36^{d}	2.25^{bc}	14.18^{fgde}	11.88^{eifhjg}
GS-32	215.66^{v}	7.56^{w}	2.58^{r}	0.0231^{n}	83.60^{ih}	0.0120^{no}	341.48^{g}	2.00^{c}	13.75^{fgeh}	$12.13^{eidfhcg}$
GS-33	166.57^{z1}	5.67^{z1}	2.07^{v}	0.0217^{r}	80.39^{l}	0.0124^{k}	365.33^{e}	2.00^{c}	12.33^{kijh}	11.35^{ikfhjg}
GS-34	140.95^{z2}	4.75^{z2}	1.73^{x}	0.0214^{s}	81.58^{kj}	0.0123^{lm}	364.16^{e}	2.50^{bc}	13.50^{fgieh}	11.58^{eifhjg}
LSD ($P \leq 0.05$)	1.66	0.07	0.06	0.0002	1.18	0.0002	4.20	0.56	1.62	1.26

Superscript in small letters on the value of each leaf sclerophylly parameter indicates significant difference at $P < 0.05$.

study are in view with the findings reported earlier by Kok (2011) in grape cultivars Cabernet Sauvignon and Merlot, in mangosteen (Qosim *et al.* 2011) and in Kinnow mandarin (Mallick *et al.* 2016).

Differences in gas exchange parameters like photosynthetic rate (*A*), transpiration rate (*E*), internal carbon dioxide concentration (*Ci*) and stomatal conductance (*gs*) recorded in the mutated population are presented in Fig 1. Rate of photosynthesis was significantly reduced in the mutants during the period under observation, i.e. April. As compared to wild type, reduced photosynthetic rate (*A*) without any statistical variation were recorded in mutants GS-25 (30 Gy), GS-31 and GS-32 (35 Gy). Rate of transpiration (*E*) was higher in GS-1, whereas internal carbon dioxide concentration (*Ci*) was significantly higher in mutants GS-25 and GS-33. Mutant GS-34 developed from higher irradiation doses transpired least whilst the *Ci* value was minimum in GS-17. A significant decrease in stomatal conductance (*gs*) was measured in mutant GS-32 and GS-34 and was statistically similar to GS-9 (15 Gy) and GS-23 (25 Gy).

Leaf gas exchange parameters measured in terms of photosynthesis, stomatal conductance and transpiration showed significant effect of radiation treatment. The doses between 30-35 Gy inhibited the photosynthetic rate, while reduced stomatal conductance was observed in some of the mutants developed from 15, 25 and 35 Gy. It is logical to imply that the lower photosynthetic and stomatal conductance in the mutants at higher radiation dose may be a consequence of disturbances in chloroplast function thus inhibiting the enzymatic activities of chlorophyll biosynthesis and CO_2 fixation. Similar decline in photosynthetic rate of mutants developed from higher irradiation doses have been reported in Kinnow mandarin (Mallick *et al.* 2016) in *Centella asiatica* (Moghaddam *et al.* 2011). Higher transpiration in the mutant GS-1 may be attributed to better leaf area, stomatal conductance and free radical scavenging in the investigated doses which however, lacked in the mutants developed at higher irradiation. The findings are in consonance with Nobel (1999) who reported that transpiration rate is greatly affected by stomatal conductance.

The simple correlations of coefficient among the leaf sclerophylly and stomata parameters of putative sweet orange cv. Mosambi mutants along with WT were calculated (Table 2). Leaf area showed significant positive correlation with the fresh weight (r= 0.92), dry weight (r=

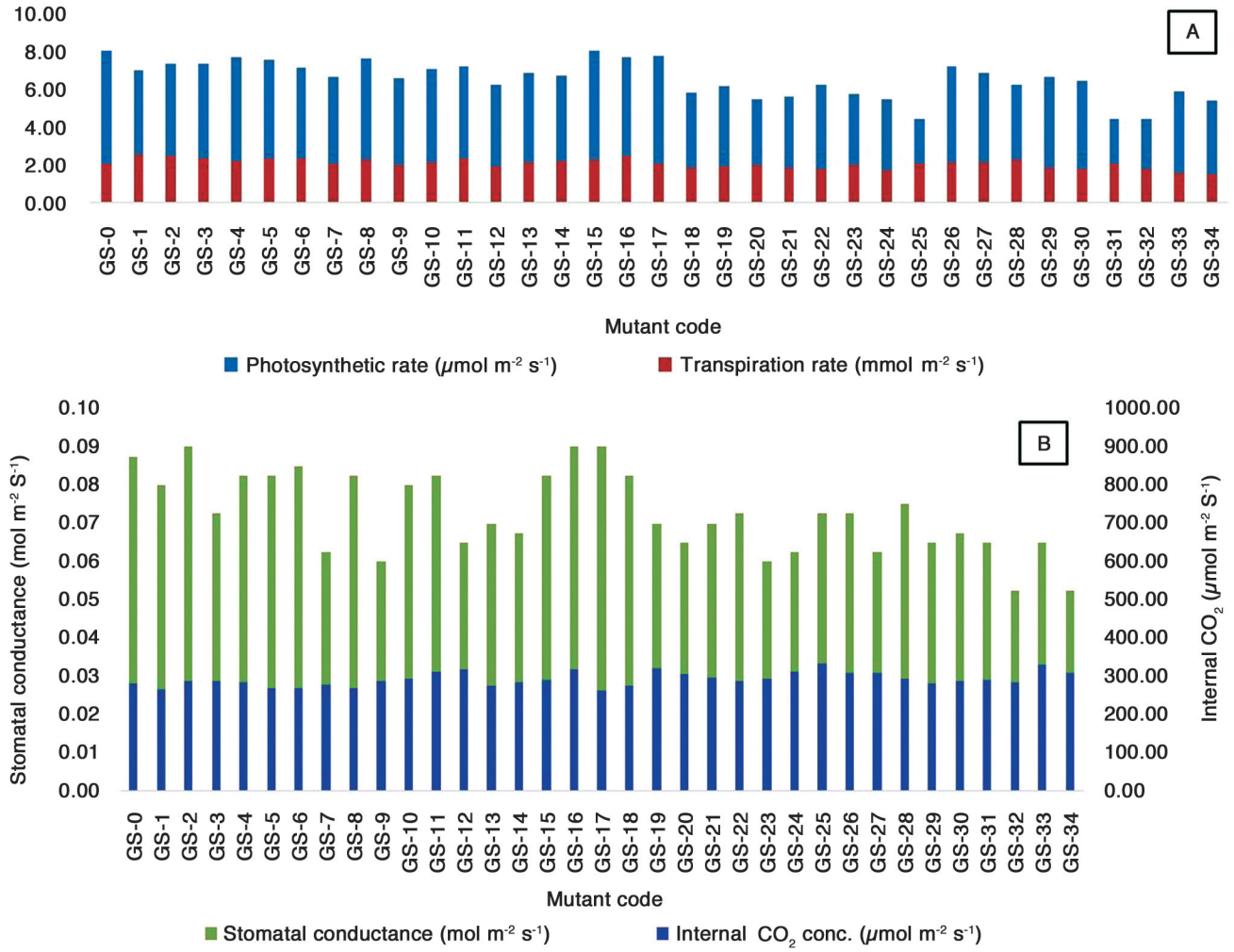

Fig 1 (A) Alteration in Photosynthetic rate (*A*) and Transpiration rate (*E*). (B) Stomatal conductance (*gs*) and internal carbon dioxide concentration (*Ci*) of γ-rays induced mutants.

Table 2 The Pearson correlation of coefficient among the leaf sclerophylly and stomata parameters of cultivar Mosambi mutants

Parameter	Leaf area	Fresh weight	Dry weight	SLA	SLW	DFT	Succulency	Stomata density	Length (μm)	Width (μm)
Leaf area	1									
Fresh weight	0.92**	1								
Dry weight	0.87**	0.88**	1							
SLA	0.39*	0.22	-0.08	1						
SLW	-0.42*	-0.23	0.07	-0.98**	1					
DFT	-0.34*	-0.50**	-0.04	-0.62**	0.60**	1				
Succulency	0.01	0.36*	0.04	-0.08	0.10	-0.72**	1			
Stomata density	0.20	0.12	0.16	0.01	-0.04	0.13	-0.16	1		
Length (μm)	0.17	0.20	0.15	0.09	-0.11	-0.18	0.13	-0.43*	1	
Width (μm)	0.04	0.14	-0.01	0.05	-0.07	-0.30	0.33	-0.26	0.67**	1

**Correlation is significant at the 0.01 level; * Correlation is significant at the 0.05 level.

0.87), specific leaf area (r=0.39); while it was significantly negatively correlated with specific leaf weight (r= -0.41) and density of foliage tissue (r= -0.34). Leaf fresh weight was significantly and positively correlated with leaf dry weight (r=0.88), succulency (r=0.36) and negatively correlated with density of foliage tissue (r= -0.50). Specific leaf area showed the complete negative correlation with specific leaf weight (r= -0.98) and density of foliage tissue (r= -0.62). Specific leaf weight was significantly and positively correlated with density of foliage tissue (r= 0.60). Density of foliage tissue showed significant negative correlation with succulency (r= -0.72). Stomata density was negatively correlated with stomata length (r= -0.43). Stomata length showed positive correlation with stomata width (r= 0.67). The other parameters were not significantly correlated for the present set of sweet orange cv. Mosambi mutants.

Results obtained from this study showed that sweet orange cultivar Mosambi developed from different doses of gamma radiation resulted in considerable variability of traits which needs to be further studied for its use in sweet orange improvement programme. The study also gave the illusion that within the same dosimetry of the mutated population, both stimulatory and inhibitory changes may take place in the morphological, physiological and anatomical characteristics. Thus, conclusively it cannot be said that high or low radiation cause only deleterious or beneficial effects as it can be *vice-versa* and hence the population should be critically evaluated for different traits to develop superior mutant for future improvement programmes.

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