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Inducing Mutations in Baladi Orange By Irradiation

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Abstract: It is well known that seedless fruit is an important economic trait relating to fruit quality. Seedless varieties are preferred for both consumers and the processing industry. Gamma irradiation is a common tool used to obtain seedless citrus fruits; hence, it was possible to obtain completely seedless fruits by irradiation with gamma rays at a dose of 20 Gy. The results showed a significant similarity in most fruit traits between the original cultivar and the new genetic composition, except for the presence of seeds and some other traits such as juice sugar content and acidity. The genetic study using molecular biology tools confirmed this genetic variability through the recorded data analysis of (SCoT and ISSR) PCR products which revealed a total of 14 polymorphic bands (8 and 6, for each, respectively) with a total polymorphism percentage of 20.89 % (22.85 % - 18.75 %, for each, respectively). This work aimed to obtain new genetic structures of the Egyptian baladi orange (*Citrus sinensis, Osbeck*), focusing on the trait of seedless fruits while preserving as much as possible the other distinctive characteristics of the original Egyptian baladi orange cultivar. Generally, it can be say that this irradiated plant material is considered as an introduction of a new seedless strain of Egyptian baladi orange.

Key words: Seedless fruits % Fruit quality % Egyptian baladi orange % Irradiated treatments % SCoT and ISSR Molecular Markers

INTRODUCTION

Of all cultivated citrus fruits, sweet oranges are the most important for many consumers. Nowadays, in Egypt sweet orange industry mainly depends on many varieties. There are two early varieties, navel and succari orange, mid-season baladi and blood orange and the Valencia orange at the end of the season. For a long time, sweet baladi orange consider the most popular varieties, especially in the upper Egypt and despite the great variation in the characteristics of its trees from one orchard to another, as most of these plants were of different strains brought from the Upper Egypt region as a seedling trees, which led to many of differences in the ability of these plants to grow and produce a homogeneous varieties that spreads and competes with the other orange varieties.

Under Egypt conditions sweet orange [*C. sinensis* (L) Osbeck] cv. "Baladi" cultivar grown in and is well acclimatized to local conditions, where acidity and sweetness are well balanced, giving them an excellent taste. The peel is smooth and shiny. the fruiting season spans from December to late February. whereas, sweet oranges like Succari, navel, and blood orange are mainly consumed fresh, while a smaller percentage is processed into value-added products. It has abundant juice content of high quality. Therefore, the majority of Egyptians consider the baladi orange ranked to the first type of juice in compared Valencia orange which widely cultivated in Egypt. In spite of baladi orange fruits have a large number of seeds which reduce the fruit juice quality by increasing of many compounds that lead to bitterness, sticks and its astringent taste. Even if the fruits are used for fresh consumption, yet the large number of seeds remains as an undesirable characteristic for the consumer. Bearing in mind that citrus fruits with less than 5 seeds are considered as seedless fruits [1].

Therefore, the idea of improving the Egyptian baladi orange through producing an genetic strain that carries the same characteristics as before, provided that its fruits are seedless and genetically identical.

To date, gamma radiation mutagenesis of budwood has been the most commonly used method by citrus breeders around the world to obtain clones without seeds

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of commercial seed varieties [2, 3]. For example, gamma radiation mutations have been previously applied to achieve new seedless varieties of oranges, mandarins, grapefruits, and lemons thus, mutation through irradiation can enhance the frequency of seedless mutants [4, 5]. Another way to obtain seedless citrus fruit is by breeding triploid trees, but this method presents several drawbacks, such as prolonged juvenility and trees with long thorns and small vigor size [6]. Unlike other mutagenesis methods, gamma irradiation at the doses used to induce seedlessness is a rather drastic procedure. Herein , we report a study of a new seedless baladi orange clone obtained by gamma irradiation.

This study aimed to produce a genetic strain of Egyptian baladi orange that carries the same characteristics of the mother plant, provided that its fruits are seedless and genetically identical in all its plants.

MATERIALS AND METHODS

Several well-growing baladi orange trees (*Citrus sinensis* L.Osbeck) were selected (season 2016), free of apparent diseases and pests, enjoying good annual production and high-quality fruits from a private farm at the Nubaria region. Then, 200 scions were selected from the previous trees, kept in a wet-peat moss and directly taken to the National Research Center to be exposed to radiation doses, the appropriate (20 & 40 Gy) doses and different periods . After that, these scions were grafted onto a suitable rootstock ‘volkamer lemon" (*C. volkameriana*) that had been planted in advance for the grafting process (season 2016). Grafted trees were cared for two growing seasons (2016 & 2017) by removing all the suckers on the trees until we obtained good, fruitful growth coming from only one bud from each scion. With attention has been paid to irrigate and fertilize those trees and carrying out needed management with appropriate control for all previous periods. In the 3rd season (2018), tree gave only one, fruitful and distinct scion irradiated by dose 20 Gy and obviously had seedless fruit was selected to take grafting scions from it and graft them onto other volkamer lemon trees that were specially planted to install those scions on them. Horticultural practices were carried out for those trees until they became fruitful in the following seasons. At 2021-2022 & 2022-2023 seasons, the fruits of those trees were taken and compared with the original Egyptian baladi orange trees from which the original graft was taken before the irradiation process was carried out. This means that the actual data of this experimental work were recorded in 2021-2022 and 2022-2023 seasons.

The following criteria were used to assess the tested treatments:

Fruit Characteristics:

Fruit Physical properties: Samples of 24 fruits /replicate were randomly taken (8 fruits for each tree), the studied parameters involved: average fruit: weight (g), volume (cm^3), height & diameter (cm), peel thickness(mm), and juice weight (g).

Number of Segments and Seeds: The number of segments and seeds per fruit was recorded by cutting the fruits in a half and extracting the juice with a hand extractor.

Fruit Juice Properties: The following criteria were taken into account: Juice ascorbic acid content (mg/100 ml) was determined by 2, 6-dichlorophenol-indophenol titration (mg/100 ml) following AOAC method [7]. The total soluble solids (TSS%) was measured by a hand-refractometer. Also, total acidity as citric acid (g/100 ml) was determined by sodium hydroxide titration (0.1 N) by using phenolphthalin reagent, and the TSS/acid Ratio was calculated.

Molecular Genetic Analysis:

DNA Isolation: Genomic DNA was isolated from fresh leaves of citrus by DNeasy plant mini kit (bio basic). DNA quality was checked utilizing absorbance ratios A_{260}/A_{280} through a UV-spectrophotometer where DNA is pure with a ratio A_{260}/A_{280} from 1.8- 2.0. Moreover, a qualitative check for DNA samples was done using electrophoresis in 1.5% agarose gel with ethidume bromied.

Polymerase Chain Reaction: Genomic DNA was used as a template for Polymerase Chain Reaction (PCR) amplification using 7 SCoT primers and 7 ISSR primers in molecular analysis for the cultivar and treated one. ISSR primers procured from Operon Technology, Alameda, U.S.A. On the other hand, SCoT and ISSR primers were designed from a consensus sequence derived from the previous studies by Joshi *et al.* and Collard *et al.* [8, 9] and procured from Biobasic Com. All SCoT primers were 18-mer and were from Dataset I which is based on highly expressed genes as described by Sawant *et al.* [10]. For SCoT primers design, the start codon ATG (+1, +2, and +3), ‘G’ at position +4, ‘C’ at position +5, and A, C, C, and A at positions +7, +8, +9 and +10, respectively, were fixed (5′-----ATGGCTACCA---3′). Amplification reactions for the SCoT technique was performed as described by Fathi *et al.* [11] and Xiong *et al.* [12] respectively, and were carried out in Techni TC-512 Thermal Cycler as follows: One cycle at 94°C for 4 min followed by 40 cycles of 1 min

at 94°C, 1 min at annealing temperature 57°C and 2 min at 72°C, followed by 72° C for 10 min, the reaction was finally stored at 4°C.

Gel Electrophoresis: Amplified products were loaded and separated on a 1.5% agarose gel with ethidume bromied and 100 bp to 1.5 kb ladder markers. The run was carried out for about 30 min at 100 V in mini submarine gel BioRad.

Gel Reading and Analysis: DNA banding pattern photos were photographed using Bio-1D Gel Documentation system. They were analyzed by GelAnalyzer3 software which scored clear amplicons as present (1) or absent (0) for each primer and entered in the form of a binary data matrix. From this matrix, DNA profiles were performed for SCoT techniques according to Adhikari *at al.* [13].

Statistical Analysis: A Completely Randomized Design (C R D) with3 replicates with 3 trees for each replicate was used. The data obtained were statistically analyzed using the analysis of variance method, as reported by Snedecor and Cochran [14]. The differences between means fruit from gamma-irradiated varieties as compared to fruit of their corresponding unirradiated variety were differentiated by using LSD5% test by using the CoStat statistical software, version 6.400 and the Microsoft Office Excel program.

RESULTS AND DISCUSSION

Fruit Characteristics: Different fruit quality features have been evaluated in the irradiated baladi orange under study, it is evident by analyzing the data in Table (1) and Figure (1) that there were no statistically proven significant differences between the Egyptian baladi orange before exposure to radiation and the strain resulting from irradiation regarding the characteristics of the weight, size, and dimensions of the fruit, the peel thickness, the weight of the juice, the number of segments in the fruit, and the ascorbic acid content of the juice. This trend held throughout the two seasons of the study. While, the parameters of the seeds number in fruit and the juice acidity and TSS or the TSS acid ratio, significant differences took place between the original baladi orange trees and those that were exposed to irradiation, as the original strain was higher in the number of seeds, in other words, examined clones presented lower seed number zero vs -13.2 and 12.6 seeds/fruit, in both seasons, respectively.as for juice TSS values, they were lower than the untreated control trees, on the contrary, juice acidity obtained higher values with irradiation treatment yet, TSS / A scored higher value in the second with irradiation treatment which encourage an earlier fruit maturity. thus, the resulted fruits showed desirable fruit quality parameters in terms of their seed number, high vit. C content. These clones have the potential to gain recognition as a distinct variety. This effect may be due to the absence of seeds in the fruits, which in turn is responsible for the production of gibberellin in the fruits, which may be responsible for these results [15].

Molecular Genetic Identification: Molecular genetic markers and genetic variability among the baladi orange cultivar and its irradiated treatment cultivar under investigation were performed using SCoT and ISSR techniques with the baladi orange cultivar and its irradiated treatment cultivar, seven primers of SCoT and

Table 1: The differences of fruit physical and chemical parameters between Egyptian baladi orange cultivar and irradiated treatment.

	2021-2022 season			2022-2023 season		
	Control	Mutation	LSD 5%	Control	Mutation	LSD 5%
Fruit weight (g)	142.96a	133.16a	10.44	143.89a	135.10a	11.10
Fruit volume (cm^3)	152.97a	142.48a	11.88	153.96a	144.56a	12.14
Fruit length (L) (cm)	6.61a	6.42a	0.42	6.77a	6.58a	0.35
Fruit diameter (D) (cm)	6.42a	6.28a	0.38	6.64a	6.27a	0.42
Fruit shape index (L/D)	1.03a	1.02a	0.18	1.02a	1.05a	0.15
Peel thickness (mm)	3.14a	3.05a	0.15	3.14a	3.07a	0.11
Juice weight per fruit (g)	74.53a	72.96a	3.02	73.38a	75.05a	3.39
Juice (%)	52.13b	54.79a	1.09	51.00b	55.55a	1.25
Juice TSS (%)	12.39a	11.84b	0.31	12.26a	11.44b	0.47
juice acidity (%)	1.60b	1.80a	0.08	1.57b	1.70a	0.11
TSS / acid ratio	7.74a	6.58b	0.61	7.81a	8.29b	0.43
Ascorbic acid (mg/ 100 ml)	45.25a	46.43a	3.87	45.11a	46.78a	2.03
Numbers of seeds	13.22a	0.00b	0.79	12.67a	0.00b	1.10
Numbers of segments	10.29a	9.51a	1.82	10.81a	9.63a	1.83

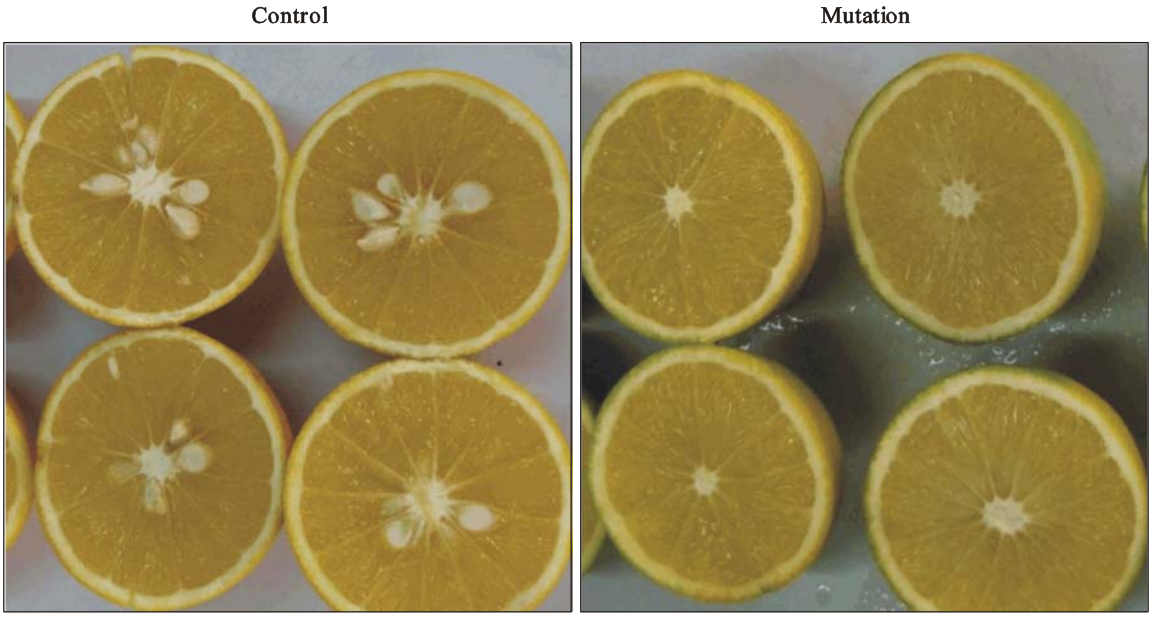

Fig. 1: A cross-section of orange fruits showing the difference in the number of seeds between the original baladi orange and the mutation resulting from irradiation.

Table 2: Banding patterns data as estimated for Egyptian baladi orange cultivar and irradiated treatment using SCoT technique.

Primer Name	M.W Range (bp)	Sequence	Total Band	Monomorphic Band	Polymorphic Band	Unique Markers	Polymorphism %
ScoT 2	320-1080	ACC ATG GCT ACC ACC GGC	3	3	-	-	-
ScoT 4	280-1120	ACC ATG GCT ACC ACC GCA	7	6	1	1	14.28
SCoT 7	760-1380	ACA ATG GCT ACC ACT GAC	3	2	1	1	33.33
SCoT 10	280-1380	ACA ATG GCT ACC ACC AGC	6	5	1	1	16.66
SCoT 11	330-760	ACA ATG GCT ACC ACT ACC	4	4	-	-	-
SCoT 12	380-1420	CAA CAA TGG CTA CCA CCG	6	4	2	2	33.33
SCoT 13	180-830	ACC ATG GCT ACC ACG GCA	6	3	3	3	59.00
		Total	35	27	8	8	22.85

Table 3: Molecular banding patterns data estimated for Egyptian baladi Orange cultivar and irradiated treatment using ISSR technique.

Primer Name	M.W Range (bp)	Sequence	Total Band	Monomorphic Band	Polymorphic Band	Unique Markers	Polymorphism %
49A	270-680	CAC ACA CAC ACA CA	4	4	-	-	-
49B	215-870	CAC ACA CAC ACA GG	7	5	2	2	28.57
89B	530-840	CAC ACA CAC ACA GT	4	2	2	2	50.00
HB-10	435-1780	GAG AGA GAG AGA CC	3	2	1	1	33.33
HB-11	240-420	GTG TGT GTG TGT TGT CC	3	3	-	-	-
HB-12	220-1180	CAC CAC CAC GC	6	6	-	-	-
HB-13	260-860	GAG GAG GAG GC	5	4	1	1	20.00
	Total		32	26	6	6	18.75

seven primers of ISSR produced bands and amplified bands of these two analyses were illustrated in Figures (2 and 3) and Tables (2 and 3).

SCoT-PCR Molecular Genetic Identification: Molecular genetic analysis of the Egyptian baladi orange cultivar and its irradiated treatment SCoT primers data were illustrated in (Figure 2 and Table 2). The results revealed that 35 bands as a total number with molecular sizes ranging from 180 to 1380 bp. The results showed 27 monomorphic bands and 8 total polymorphic bands with polymorphism percentage of (22.85 %) and the highest polymorphic percentage was recorded (59 %) produced with primer SCoT 13 and the lowest polymorphic percentage was (14.28 %) present with primer SCoT 4 was the highest in amplified bands (7 bands) and each of primers SCoT 2 and SCoT 7 were the lowest in amplified bands (3 bands). On the other hand, the results showed 8 unique markers can be used as (MAS) in genetic improvement over all the seven primers, and these results

Table 4: Polymorphic, Monomorphic, Unique Markers and Polymorphic percentage generated by SCoT and ISSR combined analysis for baladi Orange and irradiated treatment cultivars.

Primer Name	Total Band	Monomorphic Band	Polymorphic band	Unique Markers	Polymorphism %
ScoT	35	27	8	8	22.85
ISSR	32	26	6	6	18.75
Total	67	53	14	14	20.89

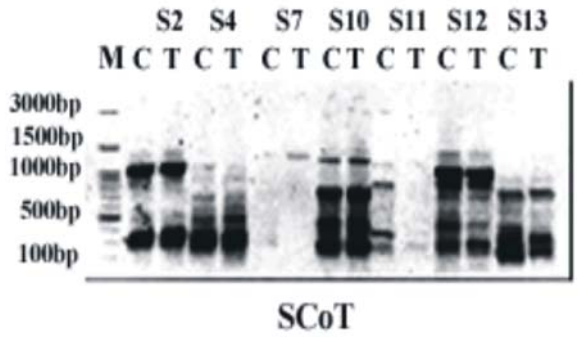

Fig. 2: Banding patterns of SCoT -PCR products for Egyptian baladi orange cultivar and irradiated treatment produced with seven primers.

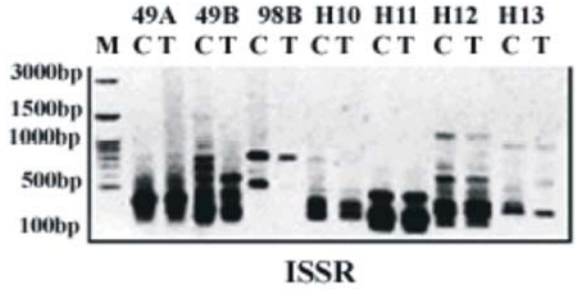

Fig. 3: Banding patterns of ISSR-PCR products for Egyptian baladi Orange cultivar and irradiated treatment produced with seven primers.

were recommended as revealed by Adhikari *at al.* [13] in Cymbopogon**;** Ahmed *et al.* [16] and Saleh *et al.* [17] in Deciduous Rootstocks and Abd El-Aziz *et al.* [18] in Apricot Rootstocks.

ISSR-PCR Molecular Genetic Identification: Figure (3) and Table (3) represented ISSR molecular genetic analysis of Egyptian baladi orange cultivar and its irradiated treatment which were obtained as a total number of bands 32 bands with molecular sizes ranging from 215 to 1780 bp. The results obtained 26 total monomorphic bands and 6 polymorphic bands with a polymorphism percentage of (18,75 %) and the highest polymorphic percentage was recorded (50 %) produced with primer 98B and the lowest polymorphic percentage was (20 %) present with primer HB-13. On the Other hand, primer 49B was the highest in amplified bands (7 bands) and each of the primers (HB-10 and HB-11) were the lowest in amplified bands (3 bands). On the other hand, the results showed 6 unique bands over all the seven primers and these results were in agreement with the finding of Gorji *et al.* [19] in Potato, Mohamed *et al.* [20] in EL Amar Apricot strains; Saleh *et al.* [17] and Baghizadeha and Dehghanb [21] in Deciduous Rootstocks.

The Egyptian baladi orange cultivar and its irradiated plant material combination data of SCoT and ISSR primers were shown in Table (4) revealed a sum of 67 band. These bands were identified as 53 monomorphic and 14 polymorphic ones with polymorphism % (20.69 %) and the polymorphic bands were scored as 14 unique markers. These results revealed that genetic variation which results as a result of irradiation treatment as a tool for genetic crop improvement and we can consider that this irradiated plant material is an introduction of a new seedless strain of Egyptian baladi orange.

CONCLUSION

so overall characteristics of the new clones could indicate that it's fruits could be c collected during January even up to late February. This will be of high importance by producing seedless oranges for the Egyptian citrus industry and for the local market and export as well as the international fresh citrus market demands high quality and seedless fruit.

The present research was conducted in order to produce seedless plants of the baladi orange cultivar in a shorter possible time by implementing radiation. These seedless plants can be further multiplied vegetatively and have the potential to be released as a distinct variety.

In conclusion, budwood irradiation is a suitable technique and unique tool to improve citrus cultivars and produce new seedless clones.

Taking into account the above mentioned results, we can conclude that a new strain of the Egyptian baladi orange was obtained by using irradiation that was confirm by genetic analysis and DNA studies. This worth noting that the resulted strain is completely similar to the original

mother tree with some differences regarding it's seedlessness. Therefore, we recommend farther studies on the new strain to assess it's environmental and horticultural needs.

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