Producing Seedless Strain of Michal Mandarin by Using Gamma Ray

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ABSTRACT

This research was conducted to obtain seedless fruits of Michal mandarin, taking into account of its distinctive characteristics. Molecular genetic evaluation among Michal mandarin cultivar and its irradiated cultivar was undertaken by using SCoT and ISSR molecular analysis for PCR reactions. The tested primers revealed a sum of 64 bands. These bands were identified as 53 monomorphic and 11 polymorphic ones with polymorphic (17.18 %) and the polymorphic bands were scored as 11 specific markers. The results showed occurrence of genetic variation according to irradiation treatment that produced eight of positive selected markers were absent in Michal mandarin cultivar and these positive markers may be linked to a new trait and thus we can consider that the irradiated strain from Michal mandarin is a new strain. Moreover, the results demonstrated a considerable resemblance in the majority of fruit attributes between the original cultivar and the new genetic makeup, except for the presence of seeds and a few other characteristics like fruit weight, fruit volume, peel thickness, ascorbic acid.it is suggested that, this new irradiated plant material can be considered as an introduction to the new seedless Michal mandarin strain. Hence, seedless fruits could be produced by irradiating them with 20 Gy of gamma rays.

Keywords: Seedless Michal mandarin- Irradiation- SCoT and ISSR Molecular Markers

INTRODUCTION

Michal mandarin is considered to be a spontaneous hybrid of the clementine and Dancy tangerine; it ripens early and is easy to peel and adapts well to the warm climate. Unfortunately, this cultivar has three disadvantages: marked alternate bearing with considerable fluctuations between heavy and light crops; a strong tendency to produce small fruits (50-60mm diameter); variable seed content, usually more than six seeds per fruit. When the fruits are used for fresh eating, the large number of seeds unfavorable to the consumer. In contrast citrus fruits with less than 5 seeds are considered seedless fruits Grosser (1998) and El-Harouny et al. (2024). Therefore, the idea was to produce a new Michal mandarin strain that carries the same characteristics of the original variety with a seedless fruit. To date, gamma radiation mutagenesis of bud wood has been the most commonly used method by citrus breeders around the world to obtain clones without seeds of commercial seed varieties El-Harouny et al. (2024), Gidoni and Carmi (2007) and Vardi et al. (2008). For example, gamma radiation mutations have been previously applied to achieve new seedless varieties of oranges, mandarins, grapefruits and lemons El-Harouny et al. (2024), Spiegel-Roy et al. (1985) and Roose and Williams (2007). 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 El-Harouny et al. (2024) and Aleza et al. (2012). Unlike other mutagenesis methods, gamma irradiation at the doses used to induce seedlessness is a rather drastic procedure.

To study DNA, several molecular markers have been adopted such as random amplified polymorphic DNA (RAPD); inter-simple sequence repeat (ISSR), simple sequence repeats (SSRs), amplified fragment length polymorphism (AFLP), restriction fragment length polymorphism (RFLP), coding and non-coding regions of chloroplast DNA, internal transcribed spacer (ITS) region etc. For the analyses of genetic diversity, relationships, cultivars identification, linkage mapping, and molecular phylogeny in Citrus Jena et al. (2009). Among the molecular markers, RAPD and ISSR marker have extensively

been used to study genetic diversity and relationships in Citrus species Digvender et al. (2013), Etminan et al. (2016) and Aswathy et al.(2017). SCoT is superior over other dominant DNA marker systems like RAPD and ISSR in higher polymorphism and better marker resolvability Mohamed et al. (2015) in El-Amar apricot strains Abd El-Aziz and Rehab (2016) in canolla; Abd El-Aziz et al. (2016) in tomato and Abd El-Hadi et al. (2017) in squash; Awad et al. (2018) in some local Apricot lines; Safaa et al. (2018) in deciduous rootstocks and Abd El-Aziz et al. (2019) in apricot rootstocks.

Therefore, the aim of this work was to produce a seedless new Michal mandarin strain that carries the same characteristics of the original variety with a seedless fruit and all its plants are genetically identical as much as possible.

MATERIALS AND METHODS

In an extension of a previous research work, that included several well-growing selected Michal mandarin trees (season 2016), free of apparent diseases and pests, enjoying good annual production and high-quality fruits from a private farm in the Nubaria area, 50 budsticks were selected from the aforementioned trees then placed in peatmoss, then taken directly to the National Research Center to expose to radiation at the appropriate dose (20 and 40 Gy). After that, these budsticks were grafted onto trunk of old Volkamer lemon *(Citrus volkameriana)* trees (4 budsticks per tree), and the grafted trees were cared for two full seasons (seasons 2016 and 2017), removing all the suckers on the trees until we obtained good, fruitful growth coming from only one bud have a seedless fruits, irrigating and fertilizing were carried out to those trees and carrying out operations. Appropriate control for all previous periods. In the third season (season 2018), the growth produced of 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 Volkamer lemon *(Citrus volkameriana)* seedlings rootstocks that were specially planted for budding process, the young trees were taken care until they bore fruit in the following years. In the 2021-2022 and 2022-2023 seasons, the fruits of those trees were taken and compared with the original Michal mandarin trees from which the original graft was taken before the irradiation process was carried out. Thus, the actual data of this experimental work were recorded at 2021-2022 and 2022-2023 seasons

The following criteria were used to assess the tested treatments

A-Characteristics of fruits: -

A-1-Physical fruit properties

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

A-2-Number of segments and seeds

The number of segments and seeds per fruit was recorded after cutting the fruits in half and extracting the juice with a hand extractor.

A-3- Chemical Fruit Properties

The following attributes were taken into account: Juice ascorbic acid content (mg/100 ml) was determined using 2, 6-dichlorophenol-indophenol titration (mg/100 ml) following **AOAC (2016)** method. The total soluble solids (TSS %) was determined using a hand-refractometer. Also, total acidity as citric acid (g/100 ml) was got by sodium hydroxide titration (0.1 N) in the phenolphthalin presence as indicator and the TSS/acid ratio was estimated.

B-Molecular genetic analysis: -

B-1-DNA isolation

Genomic DNA was isolated from freshly leaves of original Michal mandarin and irradiated plants by DNeasy plant mini

kit (bio basic). The DNA pellet was dissolved in 100 µl of TE buffer. The extracted DNA was quantified using Thermo Scientific Nano Drop™ 1000 Spectrophotometer and 0.8%

B-2-Polymerase Chain Reaction

Genomic DNA was used as a template for Polymerase Chain Reaction (PCR) amplification using 7 SCoT primers and 6 ISSR primers in molecular analysis for the cultivar and treated one PCR amplification of 10 µl total volume was performed in 4.0 µl of 10X PCR buffer, 1.0 µl of each of forward and reverse primer (5 µM), 3.0 µl of DNA (50 ng) and 4.0 µl distilled de-ionized water using an Eppendorf thermal cycler. The PCR profile consisted of initial denaturation at 94 °C for 4 min and subsequent 35 cycles each with denaturation at 94 °C for 1 min, primer annealing at 57 °C for 1 min and primer extension at 72 °C for 1 min. The final extension step was performed at 72 °C for 7 min. Annealing temperature was modified to optimize the reaction conditions for individual primers.

On the other hand, SCoT and ISSR primers were designed from consensus sequence derived from the previous studies by Collard and Mackill (2009). All SCoT primers were 18-mer and were from Dataset I which based on highly expressed genes as described by Sawant et al. (1999). 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 SCoT technique was performed as described by Fathi et al. (2013) and Xiong et al. (2011) respectively,

B-3-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.

B-4-Gel reading and analysis

DNA banding pattern photos were photographed using Bio-1D Gel Documentation system and were analyzed by GelAnalyzer3 software which scoring 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 et al., (2015)**.

C-Statistical Analysis

A Completely Randomized Design (C R D) with three replicates and 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 (1989). The differences between means fruit from gamma-irradiated strain as compared to fruit of their corresponding unirradiated cultivar were differentiated by using LSD5% test by using the CoStat statistical software, version 6.400 and the Microsoft Office Excel program.

RESULTS AND DISCUSSION

A-Characteristics of fruits:

According to dimensions, fruit Juice percentage, Juice TSS, Juice acidity, TSS acid ratio and numbers of segments, there were no statistical significant variations between the Michal Mandarin before radiation exposure and the strain brought on by radiation, according to the analysis of the data in **Table (1)** and **Fig. (1)**. This was verified throughout the two seasons. While significant differences were found between the original Michal mandarin trees and those exposed to irradiation in terms of fruit weight, fruit volume, peel thickness, ascorbic acid, and number of seeds. The original cultivar had more seeds, fruit weight, fruit volume, peel thickness, and ascorbic acid, all of which were confirmed during the study seasons. This effect might stem from the fruits' lack of seeds, which causes the fruits to produce gibberellin, which could be the cause of these outcomes. El-Harouny et al. (2024) and Giovannoni (2001).

Table (1). The variations in fruit physical and chemical characteristics between the irradiation treatment and the Michal Mandarin cultivar.

	2021-2022 season			2022-2023 season		
	Control	Mutation	LSD 5%	Control	Mutation	LSD 5%
Fruit weight (g)	96.76a	93.23b	2.16	95.91a	91.92b	2.72
Fruit volume (cm^3)	86.80a	79.72b	4.49	89.20a	80.06b	7.88
Fruit length(L) (cm)	5.33a	5.11a	0.23	5.40a	5.14a	0.27
Fruit diameter (D) (cm)	5.65a	5.57a	0.10	5.68a	5.55b	0.09
Fruit shape index (L/D)	0.94a	0.92a	0.04	0.95a	0.93a	0.04
Peel thickness (mm)	2.84a	2.57b	0.17	2.80a	2.57b	0.15
Juice weight per fruit (g)	42.47a	42.92a	0.67	43.05a	42.80a	1.14
Fruit Juice %	43.90b	46.04a	1.12	44.90b	46.57a	1.56
Juice TSS (%)	11.39a	10.98a	0.78	11.57a	10.70b	0.63
juice acidity (%)	0.68a	0.64a	0.05	0.69a	0.62b	0.05
TSS / acid ratio	16.69a	17.16a	0.75	16.82a	17.20a	0.73
Ascorbic acid (mg / 100 ml)	32.23a	30.78b	0.93	33.51a	32.41b	1.06
Numbers of seeds	7.90a	0.00b	1.18	8.78a	0.00b	0.80
Numbers of segments	8.74a	8.68a	1.50	8.74a	9.20a	1.14

Control **Mutation**

Fig. (1). A cross-section of orange fruits showing the difference in the number of seeds between the original Michal mandarin and the mutation resulting from irradiation.

B- Molecular genetic evaluation of Michal mandarin cultivars:

Evaluating the genetic molecular markers for the Michal mandarin cultivar and its irradiated strain using SCoT and ISSR markers. Seven SCoT and six ISSR primers gave reproducible bands and these primers were selected for final amplification and data analysis. Banding patterns and DNA profiles of these techniques were shown in **Figs. (2 and 3)** and **Tables (1, 2, 3 and 4).**

SCoT-PCR molecular genetic evaluation:

Molecular genetic analysis of the Michal mandarin cultivar and its irradiated strain under investigation, SCoT primers were illustrated **(Fig. 2 and Table 2)** 40 bands as a total number with molecular sizes ranged from 235 to 1860 bp. The results obtained 7 total polymorphic bands with polymorphic percentage of (17.50 %) and the highest polymorphic percentage was recorded (60 %) produced with primer SCoT 7and the lowest polymorphic percentage was (20 %) present with primer SCoT 12. While, primers (SCoT 13) were the highest in amplified bands (8 bands) and primers SCoT 2 and SCoT 10 were the lowest in amplified bands (4 bands). On the other hand, the results showed a 33 of monomorphic bands and 7 specific markers over all the seven primers and these results agreed with Awad et al. (2018) in some

local Apricot lines**;** Safaa et al. (2018) in Deciduous Rootstocks and Abd El-Aziz et al. (2019) in Apricot Rootstocks.

A total number of 7 Specific markers were identified by using SCoT primers as shown in Table 3. These markers ranged in size from. 370 to 1370 bp., only one of them was positive marker which detected with Michal mandarin SCoT 12 (980bp), and six were negative markers SCoT 2 815bp, SCoT 7(1370, 1060 and 780bp) and SCoT 11(830 and 370bp). On the other hand, three primers were selected positive markers with Irradiated strain (ScoT 2 815bp), SCoT 7 (1370, 1060, and 780bp) and SCoT 11(8390 and 370bp). While one selected negative markers with Irradiated strain with primer SCoT 12 980bp.

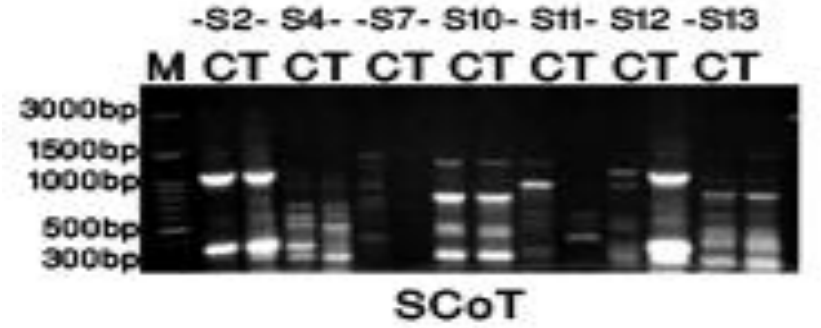

Figure (2). Banding patterns of SCoT -PCR products for Michal mandarin cultivar and its irradiated strain produced with seven primers.

Table (2). Banding patterns data as estimated for Michal mandarin cultivar and its irradiated strain using SCoT technique.

Primer Name	M.W Range(bp)	Sequence	Total Band	Monomorphic Band	Polymorphic Band	Specific Markers	Polymorphic %
SCoT 2	320-1060	ACC ATG GCT ACC ACC GGC	4	3	1	1	25 %
SCoT 4	240-1080	ACC ATG GCT ACC ACC GCA	7	7	-	-	-
SCoT 7	460-1630	ACA ATG GCT ACC ACT GAC	6	3	3	3	60%
SCoT 10	275-1680	ACA ATG GCT ACC ACC AGC	4	4	-	-	-
SCoT 11	315-1680	ACA ATG GCT ACC ACT ACC	6	4	2	2	33.33 %
SCoT 12	235-1470	CAA CAA TGG CTA CCA CCG	5	4	1	1	20.0 %
SCoT 13	320-970	ACC ATG GCT ACC ACG GCA	8	8	-	-	-
Total			40	33	7	7	17.50 %

Table (3): Michal mandarin cultivar and its irradiated strain characterized by positive and negative specific markers with their molecular sizes (bp) and total number of markers for each cultivar using SCoT analysis

Cultivars	Marker Type	Positive Specific Markers			Negative Specific Markers		
		Primer	Mol. Size(bp)	No	Primer	Mol. Size(bp)	No
Michal mandarin	**SCoT**	-	-	-	SCoT 2	815	1
		SCoT 12	980	1	SCoT 7	1370, 1060, 780	3
		-	-		**SCoT 11**	830, 370	2
Irradiated		SCoT 2	815	1	-	-	-
		SCoT 7	1370, 1060, 780	3	SCoT 12	**980**	1
		SCoT 11	830, 370	2	-	-	-
Total				7			7

ISSR-PCR molecular genetic evaluation:

Fig. (3) and **Table (4)** represented ISSR molecular genetic analysis of the Michal mandarin cultivar and its irradiated strain which were obtained as a total number of bands 24 bands with molecular sizes ranged from 185 to 925 bp. The results obtained four total polymorphic bands with polymorphic percentage of (16.66 %) and the highest polymorphic percentage was recorded (40 %) produced with primer 49B and the lowest polymorphic percentage was (20 %) present with primer HB-13. While, primers (49B and HB-13) were the highest in amplified bands (5 bands) and primers HB-10 and HB-11 were the lowest in amplified bands (3 bands). On the other hand, the results showed a 20 of monomorphic bands and 4

specific markers over all the six primers and these results were in agreement with the finding of Mohamed et al. (2015) in EL Amar Apricot strains and Etminan (2016) in durum wheat, and Safaa et al. (2018) in deciduous rootstock**.**

A total number of 4 specific markers were identified by using ISSR primers as showed in Table 5. These markers ranged in size from 470 to 640 bp., only two of them were negative markers which detected with Michal mandarin cultivar (49B 470bp and HB-13 720bp). While two primers were selected positive markers with Michal mandarin cultivar (49B 640bp and 89B 620bp). On the other hand, only two positive specific markers were detected with irradiated strain with primers (49B 470bp and HB-13 720bp) and also two negative specific markers were detected with irradiated strain (49B 640 and 89B 620).

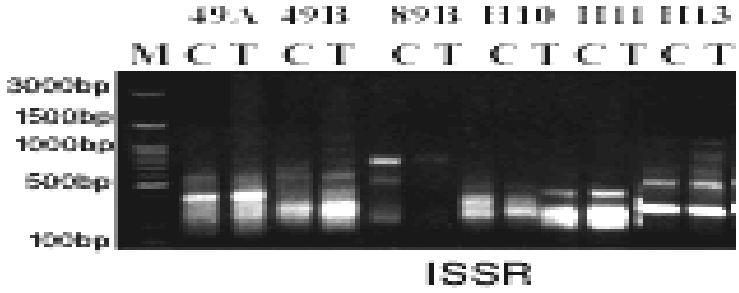

Figure (3). Banding patterns of ISSR-PCR products for Michal mandarin cultivar and its irradiated strain produced with seven primers.

Table (4). Molecular banding patterns data estimated for Michal mandarin cultivar and its irradiated strain using ISSR technique.

Primer Name	M.W Range(bp)	Sequence	Total Band	Monomorphic Band	Polymorphic Band	Specific Markers	Polymorphic %
49A	185-630	CAC ACA CAC ACA AG	4	4	-	-	-
49B	235-640	CAC ACA CAC ACA GG	5	3	2	2	40.0 %
89B	275-835	CAC ACA CAC ACA GT	4	3	1	1	25%
HB-10	330-475	GAG AGA GAG AGA CC	3	3	-	-	-
HB-11	260-530	GTG TGT GTG TGT TGT CC	3	3	-	-	-
HB-13	345-925	GAG GAG GAG GC	5	4	1	1	20%
Total			24	20	4	4	16.66 %

Table (5). Michal mandarin cultivar and its irradiated strain characterized by positive and negative specific markers with their molecular sizes (bp) and total number of markers for each using SCoT analysis

Cultivar	Marker Type	Positive Specific Markers			Negative Specific Markers		
		Primer	Mol. Size(bp)	No	Primer	Mol. Size(bp)	No
Michal mandarin	ISSR	49B	640	1	49B	470	1
		89B	620	1	HB-13	720	1
Irradiated		49B	470	1	49B	640	1
		HB-13	720	1	89B	620	1
Total				4			4

Combination evaluation of SCoT and ISSR data analysis: -

The Michal mandarin combination data of SCoT and ISSR primers showed in **Table (6)** revealed a sum of 64 band. These bands were identified as 53 monomorphic and 11 polymorphic ones with polymorphic % (17.18 %) and the polymorphic bands were scored as 11 specific markers. It possible to concluded that SCoT marker is generate from the functional region of the genome, the genetic analyses using this marker would be more useful for crop improvement programs.

Table (6). Polymorphic, Monomorphic, Specific Markers and Polymorphic percentage generated by the (SCoT and ISSR) analysis for Michal mandarin cultivar.

Primer Name	Total Band	Monomorphic Band	Polymorphic band	specific Markers	Polymorphic %
SCoT	40	33	7	7	17.50 %
ISSR	24	20	4	4	16.66 % %
Total	64	53	11	11	17.18 %

CONCLUSION

To conclude, this study showed that seedless Michal mandarin could be obtained using 20 Gy of gamma-ray irradiation. It is evident that the character is stable during the two subsequent seasons of the study.

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الملخص العربي

إنتاج سلالة يوسفي ميكال خالية من البذور باستخدام أشعة جاما

عبد الرحمن محمد عبد الرحمن حسنين و حسين سيد أحمد وسامح بهجت الحاروني و طارق علي محمود
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أُجري هذا البحث للحصول على ثمار اليوسفي ميكال خالية من البذور مع مراعة خصائصه المميزه. تم اجراء التقييم الوراثي الجزيئي بين صنف اليوسفي ميكال وسلالة المُشععه باستخدام التحليل الجزيئي SCoT و التحليل الجزيئي ISSR لتفاعلات تفاعل البوليميراز المتسلسل. كشفت البادئات المختبرة عن مجموع 64 من الحزم. وقد تم تحديد هذه الحزم على أنها 53 حزمه متشابه و11 حزمه مختلفة مع نسبة اختلاف (17.18٪) وتم تسجيل الحزم على أنها 11 علامة محددة. وقد أظهرت النتائج حدوث تباين وراثي وفقًا للمعالجة بالإشعاع حيث أن ثمانية من العلامات المختاره الإيجابية كانت غائبة في صنف اليوسفي ميكال وقد تكون هذه العلامات الإيجابية مرتبطه بسمة جديدة وبالتالي يمكننا اعتبار أن السلالة المُشععة من اليوسفي ميكال هي سلالة جديدة. علاوة على ذلك، أظهرت النتائج تشابهًا كبيرًا في معظم صفات الثمار بين الصنف الأصلي والتركيب الجيني الجديد، باستثناء وجود البذور وبعض الخصائص الأخرى مثل وزن الثمار وحجمها وسمكة القشرة وحمض الأسكوربيك. ومن المقترح أن هذه المادة النباتية المُشععة الجديدة يمكن اعتبرها مقدمةً لسلالة جديده من اليوسفي ميكال الخالية من البذور. ومن ثم، يمكن إنتاج ثمار خالية من البذور عن طريق تشعيعها بـ20 جراي من أشعة جاما.