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In vitro Multiplication and Genetic Fidelity Studies in Cleopatra Mandarin (*Citrus reshni* Tanaka)

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

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Short Research Article

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ABSTRACT

The present investigation was undertaken for *in vitro* multiplication of Cleopatra mandarin using nodal segment explants. Nodal segments taken from 9 years old plant in the month of March - April were cultured on Murashige and Skoog (1962) medium supplemented with BAP 0.5 mg/l + Kin 0.5 mg/l for establishment. Various concentrations of auxins viz. IAA and NAA were used singly or in combination with cytokinins (Kinetin and BAP) for shoot multiplication. Maximum number of shootlets per sprouted bud (8.2 ± 0.22) and maximum length of shootlets (3.0 ± 0.12cm) were recorded when auxins were used in combination with cytokinins at a concentration of NAA 0.3 mg/l + BAP 1.0 mg/l. Full strength MS media fortified with 1mg/l IBA was used for rooting of multiplied shoots and was kept in potting media containing sand, soil and vermi compost in 1:1:1 ratio for hardening.*In vitro* raised plants were examined for genetic stability by using RAPD primers. Out of fifty primers screened, eleven primers produced amplification while thirty-nine primers did not show any amplification. All RAPD profile for *in vitro* raised plants were monomorphic and similar to their mother plant, which showed that all the plants raised through micropropagation were true to type.

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Keywords: In vitro propagation; Cleopatra mandarin; citrus rootstock; nodal segments; genetic fidelity.

1. INTRODUCTION

Cleopatra mandarin, a commonly used rootstock for different cultivated species of citrus is tolerant to viruses viz., tristeza, exocortis, xyloporosis, salt, cold, and calcareous soils [1]. The plants of Sweet orange and mandarin cultivars budded on this rootstock are highly compatible. Nevertheless, the production of uniform plants in sufficient quantity is not possible through seeds owing to cross pollination, nucellar embryony, shorter viability of seeds and lesser number of seeds per fruit. It is also a slow growing rootstock in the nursery and is difficult to propagate. The importance of the citrus industry and the continuous introduction of new, improved genotypes emphasize for the use of modern methods of rapidly propagating new and promising plant materials. Tissue culture offers an advantage over conventional methods of propagation in producing large number of genetically uniform healthy plants within a short period. *In vitro* propagation ensures the availability of plant material throughout the year avoiding the necessity to import both seeds and plants. Micro propagation offers rapid multiplication of such crops in limited space and time under controlled conditions throughout the year. Among fruit species, Citrus crops are most affected by pathogens transmitted through vegetative propagation material [2]. *In vitro* propagation methods can be effectively utilised for production of disease free planting material. Micro propagation of citrus has been reported by various workers in various species viz. *Citrus jambhiri* Lush. [3], Carrizo citrange [4], *Citrus limon* L. [5] and kinnow mandarin (*Citrus nobilis* × *Citrus deliciosa*) [6]. The regeneration potential varies from species to species and thus a protocol for rapid multiplication of Cleopatra mandarin explants need to be developed.

The Genetic stability of *in vitro* regenerated plantlets is of prime importance. Somaclonal variations have been observed in plants raised through tissue culture, which defeats the purpose of producing true to type plants. The frequency of these variations varies with the species, source of explant, their pattern of regeneration (somatic embryogenesis/ organogenesis/axillary bud multiplication), media composition and cultural conditions [7]. Cleopatra mandarin may be also amenable to *in vitro* somaclonal variation. This necessitates verification of the clonal fidelity of *in vitro*-generated plants and an assessment of protocol reliability. Hence the need arises to study genetic fidelity of tissue culture raised plants using molecular marker technique to study the genetic fidelity. Most reliable method is the use of molecular markers such as RFLP (Restriction Fragment Length Polymorphism), RAPD (Random Amplified Polymorphic DNA), AFLP (Amplified fragment length polymorphism), ISSRs (Inter-simple sequence repeats), SSR (Simple sequence repeats) etc. Among these RAPD has the distinct advantage of being simple and quick to perform, requiring only small amount of DNA compared to RFLP analysis [8]. Therefore, RAPD can be used as a powerful tool for checking the genetic fidelity of *in vitro* grown plants. Taking this into account the present investigation has been planned to first multiply the plants *in vitro* and then assess the genetic fidelity of the *in vitro* raised plants through molecular markers.

2. MATERIALS AND METHODS

Explants were collected from healthy and mature plants growing at experimental orchard of Department of Horticulture, CCS HAU Hisar. The young newly emerged shoots about 10-12 cm in length were collected during morning hours (8.00 am – 10.00 am) and brought to the tissue culture lab (Centre for Plant Biotechnology, Hisar). The leaves and thorns from collected explants were removed using scalpel. These shoots were thoroughly washed under running tap water followed by washing with detergent (teepol) for 10 minutes. The explants were then given treatment with Bavistin 0.4% + streptocycline 0.2% for thirty minutes. These were then surface sterilized by treating with $HgCl_2$ 0.1% for 4min followed by dip treatment in ethyl alcohol inside the laminar flow chamber. The explants were cultured on Murashige and Skoog medium [9] supplemented with BAP 0.5 mg/l + Kin 0.5 mg/l for establishment. Various concentrations of auxins viz. IAA and NAA were used singly or in combination with cytokinins (Kinetin and BAP) for shoot multiplication. Full strength MS media fortified with 1 mg/l IBA was used for rooting of multiplied shoots. The cultures were incubated at 25 ± 2°C. After rooting the cultures were washed thoroughly with water and then these were transferred in potting media containing sand, soil and vermi compost in 1:1:1 ratio for hardening.

RAPD markers were used to test the genetic fidelity of *in vitro* grown plants with that of mother plants. For this the DNA from mother plant and 8 randomly selected *in vitro* grown plants were isolated using Cetyl Trimethyl Ammonium Bromide (CTAB) extraction method of Murray and Thompson [10], modified by Saghai-Maroof et al. [11] and Xu et al. [12]. PCR amplification was performed in PTC-100 programmable thermal cycler (MJ research and Biometra personal) in a volume of 20µl containing 2 µl of 20 ng/ µl template DNA, 2.5 µl of 10mM d NTPs mix, 1.0 µl of primer, 0.3µl of 10 X Taq DNA polymerase buffer and 0.3 µl of 5 Units/µL Taq DNA polymerase. A total of fifty primers were used for RAPD analysis to study the genetic fidelity of plants randomly selected from regenerated populations of *in vitro* plants and mother plants.

3. RESULTS AND DISCUSSION

3.1 Number of Shootlets Formed per Bud

The results for number of shootlets formed per regenerated bud derived from nodal segments of *C. reshni*, inoculated on MS medium supplemented with different concentrations and combinations of IAA and NAA singly and in combination with BAP or Kinetin (Table 1). When IAA was used singly 0.3mg/l gave maximum number of shootlets per sprouted bud (1.4 ± 0.11). NAA 0.3mg/l resulted in maximum shootlets per sprouted bud (6.0 ± 0.38) when it was used singly. On combining auxins with cytokinins maximum number of shootlets formed per bud on 7th day (2.1), 14thday (4.9) and 21stday (8.2) (Plate 1) were recorded in treatment MS + NAA 0.3 mg/l + BAP1.0 mg/l. It was significantly higher than all other hormonal treatments given for shoot multiplication. The use of BAP in combination with NAA had a promotery effect on the formation of number of shootlets. NAA concentration above 0.3 mg/l significantly reduced the number of shootlets formed. Sharma et al. [6] also found that high concentration of cytokinin and low concentration of auxin is required for shoot multiplication of Kinnow. Similar results were observed by Kumar et al. [13] in acid lime where maximum number of shoots (8.40) was recorded with BAP (MS + 0.5 mg/l) + NAA (MS + 0.5 mg/l).

3.2 Length of Shootlets

Maximum length of shootlets 1.4 ± 0.08 and 2.8 ± 0.09 cm were obtained at IAA 0.3mg/l and NAA 0.3mg/l respectively when IAA and NAA were used singly. Maximum length of shootlets (3.0 ± 0.12cm) after 21 days was recorded in treatment MS + NAA 0.3 mg/l+ BAP1mg/l where auxins were used in combination with cytokinins (Table 1). This was statistically at par with treatment MS+ NAA 0.3 mg/l). Similar results were obtained by Kumar et al. [13] who recorded maximum length of shoot in acid lime (2.65 cm) with BAP (0.5 mg/l) + NAA (0.5 mg/l). NAA was found to be better auxin than IAA while BAP was found to be better cytokinins than Kinetin with respect to both number of shootlets formed per sprouted bud and length of shootlets.

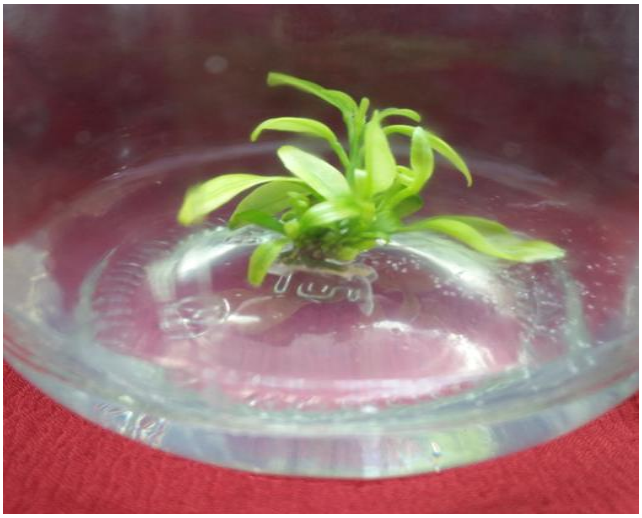

Plate 1. Shootlets from sprouted bud (MS + NAA 0.3 mg/l + BAP 1.0 mg/l)

Table 1. Effect of different hormones (auxins and cytokinin) alone and in combination on number of shootlets formed per sprouted bud at specified interval of time and shoot length

MS + Hormonal composition (mg/l)				Number of shootlets formed per sprouted bud			Length of shootlets (cm)
IAA	NAA	BAP	Kin	7th day	14th day	21st day	
-	-	-	-	0 ± 0.00	1.9 ± 0.11	2.4 ± 0.11	1.2 ± 0.12
0.1	-	-	-	1.0 ± 0.00	2.1 ± 0.11	3.3 ± 0.33	1.3 ± 0.15
0.2	-	-	-	1.2 ± 0.11	2.6 ± 0.11	3.6 ± 0.11	1.4 ± 0.04
0.3	-	-	-	1.4 ± 0.11	3.3 ± 0.00	5.0 ± 0.19	1.4 ± 0.08
0.4	-	-	-	1.3 ± 0.19	2.2 ± 0.11	3.9 ± 0.11	1.5 ± 0.15
0.5	-	-	-	1.0 ± 0.00	2.1 ± 0.11	2.7 ± 0.33	1.6 ± 0.15
-	0.1	-	-	1.8 ± 0.11	3.1 ± 0.29	4.1 ± 0.40	1.6 ± 0.05
-	0.2	-	-	2.0 ± 0.00	4.2 ± 0.22	5.1 ± 0.11	2.0 ± 0.03
-	0.3	-	-	1.9 ± 0.11	3.1 ± 0.22	6.0 ± 0.38	2.8 ± 0.09
-	0.4	-	-	1.8 ± 0.11	3.0 ± 0.19	5.1 ± 0.11	2.6 ± 0.07
-	0.5	-	-	1.1 ± 0.11	2.8 ± 0.22	3.7 ± 0.50	2.5 ± 0.12
-	0.1	1.0	-	1.7 ± 0.00	3.7 ± 0.19	5.1 ± 0.29	2.1 ± 0.04
-	0.2	1.0	-	1.9 ± 0.22	4.0 ± 0.19	6.0 ± 0.33	2.5 ± 0.21
-	0.3	1.0	-	2.1 ± 0.11	4.9 ± 0.11	8.2 ± 0.22	3.0 ± 0.12
-	0.1	-	1.0	1.3 ± 0.19	2.9 ± 0.22	4.6 ± 0.40	1.5 ± 0.10
-	0.2	-	1.0	1.6 ± 0.22	4.1 ± 0.22	6.4 ± 0.58	1.6 ± 0.00
	0.3	-	1.0	1.9 ± 0.11	4.6 ± 0.11	6.6 ± 0.48	1.6 ± 0.07
C.D. at 5%				0.37	0.51	0.96	0.32

The survival percentage of *in vitro* raised plants was maximum (90%) in potting mixture containing sand, garden soil and vermi compost in 1:1:1 ratio followed by potting mixture (80%) containing sand, garden soil and FYM in 1:1:1 ratio. Maximum per cent survival was in potting mixture containing vermi compost can be attributed to the fact that vermi compost is a finely divided, peat-like material, with high porosity, aeration, drainage, water holding capacity and microbial activity, which makes it an excellent soil conditioner [14].

3.3 Genetic Fidelity

The genetic fidelity was studied in micro propagated plants of Cleopatra mandarin by using fifty primers for RAPD analysis. Out of fifty primers screened, eleven primers produced amplification (Table 2), while thirty-nine primers did not show any amplification.

All the bands were similar and no polymorphism was found, which showed that all the plants raised through tissue culture using nodal segments as explant were true to type or identical to the mother plant (Plate 2). Although, morphological variations were found but on molecular basis, all the plants were true to type. This showed that sometimes epigenetic variation is found in tissue culture raised plants which may be due to higher level of growth regulators used in media*, in vitro* condition, number of sub culturing etc. Such epigenetic variations are not heritable and as such do not find variation in DNA [8].

Table 2. Random primers showing amplification

Sr. no.	Primer code	Sequence (5'-3')
1	LD 3244	GTGAGGCGTC
2	LD 3256	GTCCACACGG
3	LD 3257	TGGGGGACTC
4	LD 3258	CTGCTGGGAC
5	LD 3260	CCTTGACGCA
6	LD 3263	GGAGGGTGTT
7	LD 3265	AGGGAACGAG
8	LD 3266	CCACAGCAGT
9	LD 3273	GAACGGACTC
10	LD 3278	TGCGTGCTTG
11	LD 3279	CACACTCCAG

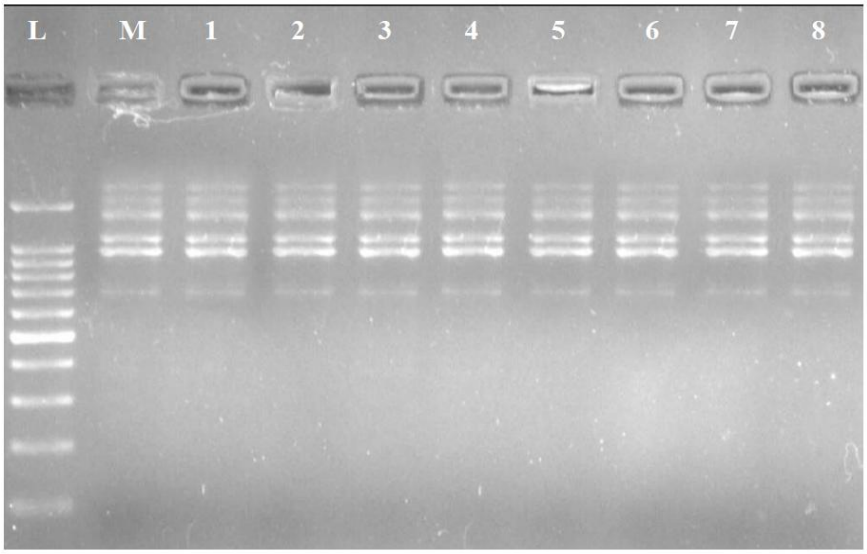

Plate 2. RAPD profiles of mother plant and *in vitro* raised plants using primer LD 3279 Lanes refer L: 100 kb ladder M: mother plant, 1-8: *In vitro* grown plants

4. CONCLUSION

The maximum number of shootlets per sprouted bud (8.2 ± 0.22) was recorded when auxins were used in combination with cytokinins at a concentration of NAA 0.3 mg/l + BAP 1.0 mg/l. Maximum length of shootlets (3.0 ± 0.12 cm) was recorded in treatment NAA 0.3 mg/l + BAP 1mg/l. Banding patterns of all tissue culture raised plants and mother plant were similar and no polymorphism was found, which showed that all the plants raised through tissue culture using nodal segments as explant were true to type or identical to the mother plant.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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