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In vitro Conservation of "Balady" Apple Shoot Tip Explants and Genetic Stability under Different Sugar Concentrations and Low Temperature

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Abstract: The goal of this study was to evaluate the *in vitro* preservation of "Balady" apple shoot tip explants and genetic stability under different concentration of sugars and low temperature. For *in vitro* preservation shoot tip explants of about 1 cm in length were excised from *in vitro* cultures and were transferred to preservation media supplemented with different concentrations of sucrose, sorbitol or glucose (10, 20, 30, 40, 50 or 60 gl^{-1}) and incubated under low temperature (5°C) for (3, 6, 9 and 12 months). All shoot tip explants conserved on media with different concentrations of sucrose, sorbitol or glucose for 3 or 6 months, on medium with 60 gl^{-1} sucrose, on medium with 50 gl^{-1} sorbitol, on media with 20 or 40 gl^{-1} glucose for 9 months, on medium with 40 gl^{-1} glucose and on medium with 50 gl^{-1} sorbitol for 12 months can be able to still alive as the survival percentage was 100.00%. On the other hand, all shoot tip explants conserved on media with 10 or 20 gl^{-1} sucrose for 12 months can not be able to still alive as the survival percentage was 00.00%. Moreover, the lowest survival percentage (33.33 and 25.00 %) respectively observed when the shoot tip explants conserved on media with 10 or 20 gl^{-1} sucrose for 9 months. An Inter Simple Sequence Repeat (ISSRs) technique was performed to detect DNA variation. No differences in the DNA fragment patterns were observed using 17 primers between samples of shoot tip explants taken before and after preservation.

Key words: Apple · *In vitro* conservation · ISSR · Low temperature

INTRODUCTION

For safe preservation, the *in vitro* slow growth storage method was developed and is considered an alternate solution for short to medium term storage of fruit germplasm. *In vitro* culture is an effective method for *ex situ* conservation of plant genetic diversity [1]. There have been two approaches to the storage of vegetatively propagated germplasm. In one, the aim is to reduce the growth rate of the cells or plants by the manipulations of culture media, reduction of incubation temperature and the combination of the former two methods. Another approaches are to stop the growth altogether [2].

Slow growth techniques, based on the manipulation of culture conditions and/or culture media, allow cultures to remain viable with a slow growth rate. Furthermore, organized culture systems for regeneration and successful propagation of genetically stable plantlets from cultures are prerequisites for *in vitro* preservation [3]. The aim of short and medium term storage is to reduce growth and to prolong subculture intervals. Slow growth has been widely applied for this purpose in fruit trees [4-8].

There are several reports of apple shoot cultures stored *in vitro*. The first report of successful *in vitro* cold storage was with the apple 'Golden Delicious', in test tubes at 1 or 4°C in the dark; shoots remained in good condition for 1 year, with some losses to contamination [9]. Single bud lines of 'Gala' apple, stored for 1 year at 4°C with a 14 h photoperiod, were studied for genetic variation. The ploidy remained stable and AFLP analysis with 20 primer pairs showed no differences in the plants before and after storage, however, some methylation changes were noted in the stored shoots [10].

This paper was conducted to study the effect of different sucrose, sorbitol and glucose concentrations (10, 20, 30, 40, 50 and 60 g/l) on the shoot tip explants of "Balady" apple conserved at 5°C under complete darkness for 12 months and ISSR-DNA analysis was used to determine the genetic stability of preserved shoot tip explants.

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MATERIALS AND METHODS

Plant Materials: Apple (*Malus domestica* L. Balady) shoot tip explants were micropropagated in glass jars contain 25 ml MS basal medium according to Murashige and Skoog [11] with 3% sucrose, 0.5 mg/l N6 benzylaminopurine (BAP) and 0.7% agar at pH 5.7. The cultures were incubated under normal growth conditions at 25 ± 2°C and photoperiods of 16 hours day and 8 hours night (light intensity of 3000 lux) and subcultured onto fresh medium every 4 weeks interval.

***In vitro* Conservation:** Shoot tip explants from "Balady" apple (1 cm long) were taken from *in vitro* cultures after 3rd subculture and separately cultured onto glass tubes (100 x 25 mm) contain MS basal medium supplemented with 0.0 mg/l BAP, 0.7% agar and different osmotic agents (sucrose, sorbitol or glucose), concentrations (10, 20, 30, 40, 50 and 60g/l) at pH 5.7. All cultures tubes were conserved at 5°C under complete darkness. Survival percentage of shoot tip explants of each treatment was recorded at the end of each conservation period (four conservation periods 3 months for each one). The experiment was arranged as factorial experiment in a completely randomized design. The obtained data were statistically analyzed according to Snedecor and Cochran [12]. Least significant difference (LSD) at 0.05 was employed to estimate the significant of differences among the treatment means.

Molecular Marker

DNA Isolation: DNA was extracted with Qiagen DNeasy kit. DNA quality was determined visually on 0.8 % agarose gel. The DNA concentration was quantitatively measured on Biophotometer (Eppendorph, Germany) and adjusted to 50 ng / µl.

PCR Reaction: PCR was performed in 25 µl reaction volume containing 2X ready mix (EmeraldAmp Max PCR master mix, 320R), 20 pM oligonucleotide primer and 25 ng genomic DNA. This reaction was performed on Eppendorf master Cycler programmed to 35 cycles as follows: an initial denaturation step at 94°C for 5 minutes, followed by 35 cycles of denaturation step at 94°C for 1 minute, annealing temperature (Ta°C) for 1 minute and an extension step at 72°C for 1 minute and final extension step at 72°C for 10 minutes.

A set of 17 ISSR primers (sequence is given in Table 1) were used, these primers were synthesised by Bioron Corporation, Germany. Ladder DNA used from (Thermo 100 bp plus). All samples were arranged from left to right. (Non conserved explants, 40 g/l sucrose, 50 g/l sucrose, 40 g/l sorbitol, 50 g/l sorbitol, 40 g/l glucose and 50 g/l glucose). Samples were applied into 2.5% agarose gel and run at Biometra horizontal gel electrophoresis device at 110 V.

Table 1: ISSR primer names, sequence and annealing temperature (Ta)

Serial number	Primer Name	Sequence (5`- 3`)	Ta (°C)
1	ISSR-1	$CAC(TCC)_5$	50°C
2	17	$CAGC(AC)_7$	50°C
3	ISSR-34	$(AG)_8TG$	53°C
4	834	$(AG)_8CT$	53°C
5	841	$(GA)_8TC$	53°C
6	ISSR-4	$CGA(CA)_7$	53°C
7	844-B	$(CT)_8$ GC	50°C
8	W844	$(CT)_8$ TG	56°C
9	15	GGTC $(AC)_7$	56°C
10	TE	GT $(GGT)_3$ GAC	47°C
11	BEC	$(CA)_7$ TC	48°C
12	17898-B	$(CA)_6$ GT	40°C
13	17899-B	$(CA)_6$ GG	42°C
14	17899-A	$(CA)_6$ AG	40°C
15	17898-A	$(CA)_6$ AC	40°C
16	3	$(CA)_8$ AT	46°C
17	CHR	$(CA)_7$ GG	51°C

Table 2: ISSR primer name, total number of amplicons, size of amplified fragments and percentage of polymorphism

Primer name	Total number of amplicons	Size of amplified fragments (bp)
1	ISSR-1	370-1418 bp
2	17	559-1397 bp
3	ISSR-34	403-831 bp
4	834	498-1550 bp
5	841	337-1659 bp
6	ISSR-4	320-1412 bp
7	844-B	439-1608 bp
8	W844	436-1608 bp
9	15	605-1402 bp
10	TE	535-1648 bp
11	BEC	657-1257 bp
12	17898-B	474-1189 bp
13	17899-B	464-1503 bp
14	17899-A	474-1818 bp
15	17898-A	604-1233 bp
16	3	552-1427 bp
17	CHR	1487-457 bp

Out of 35 ISSR primers, 17 of them gave clear pattern, all these primers were anchored either from 5 or 3, primer sequences are represented in Table 2.

Data Analysis: The banding patterns generated by ISSR primers, were compared to determine the genetic similarity of the 7 samples. Clear and distinct amplification products were scored as (1) for present and (0) for absent bands.

Bands of the same mobility were scored as identical. UPGMA used to measure the genetic similarity resulted from the analysis software of Non-Linear Dynamics Corporation (UK).

RESULTS AND DISCUSSION

***In vitro* Conservation:** The results in Table (3) show the effect of different conservation period and different sugar concentrations on survival percentage of "Balady" apple shoot tip explants conserved at 5°C under complete darkness.

Concerning to the effect of different conservation period results showed that all explants conserved for 3 and 6 months were able to still alive as the survival percentage was 100.00 %. Increasing conservation period from 6 to 9 months decreased with significant differences the survival percentage from 100.00 % to 85.18 % respectively. Also, increasing the conservation period from 9 to 12 months decreased gradually the survival percentage from 85.18 % to 73.61 % without significant differences among them.

As for the effect of different sugar concentrations on conservation media results clearly showed that conservation medium with 50 gl^{-1} sorbitol or 40 gl^{-1} glucose showed the same highest survival percentage (100.00 %). Reduction on survival percentage without significant difference was achieved when explants conserved on medium with 20 gl^{-1} glucose (97.91%), on media with 40 or 60 gl^{-1} sorbitol, 30, 50 or 60 gl^{-1} glucose which showed the same survival percentage (95.83%). Media with 30 gl^{-1} sucrose or 20 gl^{-1} sorbitol showed the same survival percentage (87.50 %) this percentage reduced to (85.42 %) on medium with 10 gl^{-1} glucose. Media with 10 or 20 gl^{-1} sucrose showed the lowest survival percentage 58.33 and 56.25% respectively, without significant differences between them.

With regard to the effect of interaction between conservation period and different sugar concentrations on conservation media results revealed that all explants conserved on media with different concentration of sugars under investigation for 3 and 6 months, the explants conserved on medium with 60 gl^{-1} sucrose, on media with 50 gl^{-1} sorbitol and on media with 20 or 40 gl^{-1} glucose for 9 months, the explants conserved on medium with 50 gl^{-1} sorbitol and on medium with 40 gl^{-1} glucose for 12 months were able to still alive as the survival percentage was (100.00%). Reduction on survival percentage from 100.00 % to 91.67 % was noticed when explants conserved on media with 40 or 50 gl^{-1} sucrose, on media with 30, 40 or 60 gl^{-1} sorbitol, on media with 10, 30, 50 or 60 gl^{-1} glucose for 9 months, on media with 40 or 60 gl^{-1} sorbitol and on media with 20, 30, 50 or 60 gl^{-1} glucose for 12 months. The survival percentage was decreased without significant difference when explants conserved on medium with 30 gl^{-1} sucrose, on media with 10 or 20 gl^{-1} sorbitol for 9 months and on medium with 40 gl^{-1} sucrose or medium with 30 gl^{-1} sorbitol for 12 months which showed the same survival percentage (83.33 %). The same survival percentage (75.00 %) was noticed when explants conserved on medium with 50 or 60 gl^{-1} sucrose and on medium with 10 gl^{-1} sorbitol for 12 months, 66.66 % when explants conserved on medium with 30 gl^{-1} sucrose and on medium with 20 gl^{-1} sorbitol for 12 months, 50.00 % when explants conserved on medium with 10 gl^{-1} glucose for 12 months and 33.33 % when explants conserved on medium with 10 gl^{-1} sucrose for 9 months. Explants conserved on medium with 20 gl^{-1} sucrose for 9 months showed the lowest survival percentage (25.00 %). While, all explants conserved on media with 10 or 20 gl^{-1} sucrose for 12 months can not be able to still alive as the survival percentage was 00.00%.

Recovery and Regeneration: After 1 year of storage, height increment of the conserved shoot tips was measured Figure 1. Subsequently, shoot tips were transferred to MS basal medium containing 0. 5 mg/l BA, 30 g/ l sucrose and 7 g/ l agar at 25°C under a 16-h photoperiod for the recovery assessment. The cultures were considered to be survival and produced axillary shoots. Shoots recovered from slow growth culture were transferred to root induction medium consisting of half strength MS basal medium, 30 g/l sucrose, 0.5 mg/l IBA, 0.5 mg/l NAA and 7 g/l agar. After promoting root initiation and development transferred and cultured on plastic pots filled with a mixture of peatmoss and sand (1:1 by volume) acclimatization for 8 weeks in the green house.

Genetic Stability Using ISSR: The DNA of the six treatments and non conserved explants of "Balady" apple was extracted and amplified using seventeen primers to estimate the genetic similarity between these treatments. All primers were successfully used as fingerprinting tool. Seventeen primers produced multiple amplicons ranged from 320 to 1659 bp. Table (2). Results presented in Figure (2) showed that each of seven samples from "Balady" apple have the genetic similarity.

Table 3: Effect of different sugar, concentrations and conservation periods (month) on survival percentage of "Balady" apple shoot tip explants conserved at 5°C under complete darkness

	Conservation period (month)				
Sugars gl^{-1}	3	6	9	12	Mean
10 sucrose	100.00 a	100.00 a	33.33 g	00.00 i	58.33 F
20 sucrose	100.00 a	100.00 a	25.00 h	00.00 i	56.25 F
30 sucrose	100.00 a	100.00 a	83.33 c	66.66 e	87.50 DE
40 sucrose	100.00 a	100.00 a	91.67 b	83.33 c	93.75 ABCD
50 sucrose	100.00 a	100.00 a	91.67 b	75.00 d	91.67 BCDE
60 sucrose	100.00 a	100.00 a	100.00 a	75.00 d	93.75 ABCD
10 Sorbitol	100.00 a	100.00 a	83.33 c	75.00 d	89.58 CDE
20 Sorbitol	100.00 a	100.00 a	83.33 c	66.66 e	87.50 DE
30 Sorbitol	100.00 a	100.00 a	91.67 b	83.33 c	93.75 ABCD
40 Sorbitol	100.00 a	100.00 a	91.67 b	91.67 b	95.83 ABC
50 Sorbitol	100.00 a	100.00 a	100.00 a	100.00 a	100.00 A
60 Sorbitol	100.00 a	100.00 a	91.67 b	91.67 b	95.83 ABC
10 Glucose	100.00 a	100.00 a	91.67 b	50.00 f	85.42 E
20 Glucose	100.00 a	100.00 a	100.00 a	91.67 b	97.92 AB
30 Glucose	100.00 a	100.00 a	91.67 b	91.67 b	95.83 ABC
40 Glucose	100.00 a	100.00 a	100.00 a	100.00 a	100.00 A
50 Glucose	100.00 a	100.00 a	91.67 b	91.67 b	95.83 ABC
60 Glucose	100.00 a	100.00 a	91.67 b	91.67 b	95.83 ABC
Mean	100.00 A	100.00 A	85.18 B	73.61 B	

Means followed by the same letter (s) in each column are not significantly different from each other at 5 % level

Fig. 1: "Balady" apple shoot tip explants on different stages.
A- Multiplication stage from shoot tip explants before storage.
B- Shoot tip explant after storage for 12 months at 5°C and complete darkness.
C- Regeneration from shoot tip explants after storage for 12 months at 5°C and complete darkness.

Therefore, no significant differences between non conserved explants and all explants conserved under different sugar concentrations at 5°C for 12 months. So these different treatments were effective for medium term storage and no genetic alteration caused by all the treatments as assayed by ISSR analysis.

Our study, in close agreement to Saker *et al*. [13] who mentioned that no significant variations were observed in tissue cultures derived date palm plantlets. This is similar to the results found by Bekheet *et al*. [14] who concluded that, plantlets derived from cryopreserved cultures were identical to that derived from non-treated cultures and both were similar with the field grown plants i.e., no genetic variability of the frozen-thawed nodular cultures of date palm. Moreover, Irina *et al*. [15] studied cold storage of *Malus domestica* 'Grushovka Vernenskaya' and *Malus sieversii* selection TM-6, they mentioned that the longest storage for 'Grushovka Vernenskaya' was 33–39 months with PGRs and 3% sucrose in either tubes or jars. TM-6 stored best in tubes on 3% sucrose with PGRs or in jars on 2% mannitol and 2% sucrose. Preliminary RAPD analysis found no significant differences between plants stored for 39 months and non-stored controls. Furthermore, Ronlize *et al*. [16] evaluate the viability of 66 accessions of pineapple plants conserved *in vitro* for 10 years by triggering resumed growth and measuring the propagative potential and genetic fidelity using 17 ISSR markers.

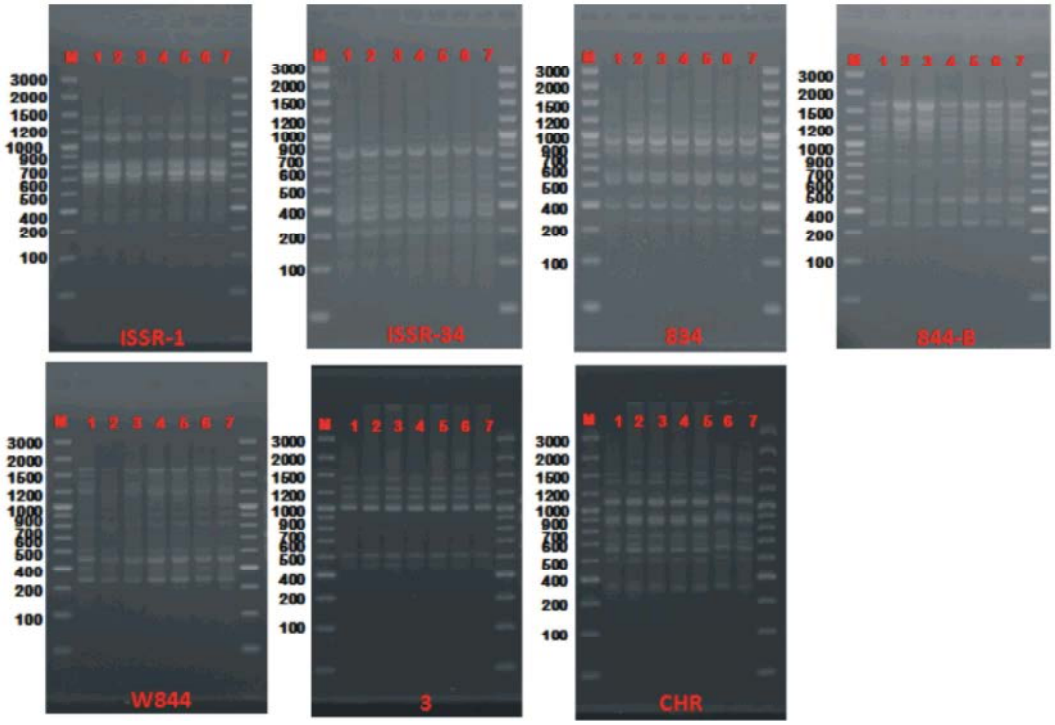

Fig. 2: ISSR profiles of "Balady" apple as detected by ISSR-1, ISSR-34, 834, 844-B, W844, 3 and CHR primers
M = 100bp marker
1- Non conserved explants
2- 40 g^{-1} sucrose 3- 50 g^{-1} sucrose 4- 40 g^{-1} sorbitol
5- 50 g^{-1} sorbitol 6- 40 g^{-1} glucose 7- 50 g^{-1} glucose

The results cleared that the accessions of the varieties A. comosus var. comosus and A. comosus var. ananassoides regenerated from plants conserved *in vitro* during 10 years remained genetically stable and two accessions of A. comosus var. bracteatus presented genetic instability, which should be better assessed in subsequent studies. A subculture interval of 24 months can be recommended for pineapple plants kept under *in vitro* conservation in Active Germplasm Banks. Moreover, ISSR markers are effective to detect somaclonal variants of pineapple plants conserved *in vitro*.

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