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ORIGINAL ARTICLES

In vitro preservation of embryogenic cultures of two Egyptian dry date palm cultivars at darkness and low temperature conditions

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ABSTRACT

This study aimed to investigate the effect of low temperature (5°C) and darkness conditions on preservation of embryogenic cultures of Bartamoda and Sakkoty date palm cultivars. *In vitro* cultures of those cultivars were obtained by culturing shoot tips excised from offshoots. Embryogenic cultures were proliferated on MS medium supplemented with 10 mg/ l 2,4-D + 3 mg/l 2iP. For low temperature preservation, cultures of the two cultivars were incubated at 5°C for twelve months. Generally, survival percentage was decreased as storage period increased in both two cultivars. After twelve months Sakkoty registered 88. 8 % and Bartamoda registered 87.7 % of survival with considerable browning specially with Bartamoda cultivar. At low temperature, rate of the new proliferated cultures of the two cultivars was decreased as increasing of storage period. Also, the effect of preservation in complete dark at room temperature conditions was investigated. It was found that survival percentage significantly declined after twelve months of preservation. Sakkoty registered high percentage of survival compared with Bartamoda cultivar. It was found that dark condition had negative effects on proliferation of embryogenic cultures in both two cultivars of date palm. Genetic stability was tested using RAPD - PCR analysis. PCR products revealed that the persevered cultures were relatively genetically stable. Bartamoda gave high similarity compared with sakkoty cultivar

Key words: Date palm, *in vitro* preservation, low temperature, darkness, RAPD analysis.

Introduction

The date palm, *Phœnix dactylifera* L., is one of the most economically important fruit tree grown in the Middle East and North-Africa. The tremendous advantages of the tree are its resilience, its requirement for limited inputs, its long-term productivity and its multiple purposes attributes (Bircher, 1990). In addition, palm tree tolerates adverse environmental conditions and it is important in reducing desertification. World production of dates was about 7 million tones and the top 10 producing countries are Egypt, Saudi Arabia, Iran, United Arab Emirates, Pakistan, Algeria, Sudan, Oman, Libya, and Tunisia (Kader and Hussein, 2009). Nowadays date palm plantations are spread out all over Egypt, wherever water is available. Date palm trees are essential components of farming system equally well in small farm units or as larger scale commercial plantation units. There are three types of varieties in Egypt based on fruit moisture i.e., a) soft varieties such as Zaghlool, Samany, Amhat and Hayani; b) semi-dry varieties such as Siwy, Amry and Aglany and c) dry varieties such as Bartamoda, Sakkoty and Malkaby. Due to the high degree of genetic heterozygosity and dioecious nature of date palm, sexual propagation method can not be used for propagation and preservation the cultivars of interest in a true-to-type manner. Conventionally, it is propagated by offshoots which arise from the base of mother plants. Date palm germplasm cannot be stored or handled easily using conventional means. At the present, the most common method used to preserve the genetic resources of date palm is as whole plants in the field. There are, however, several problems with the field gene-bank (Engelmann, 1991). The collections are exposed to natural disasters and attack by pests and pathogens; moreover, labor cost and the requirements for technical personal are very high. In addition, distribution and exchange from field gene-bank is difficult because of the vegetative nature of the material and the greater risk of disease transfer. There is a great need to alternative methods of propagation and conservation of date palm cultivars in Egypt.

Plant tissue culture techniques have been developed to set storage methods of plant germplasm. Preservation of the plant cells, meristems and somatic embryos has become an important tool for the storage of germplasm using minimum of space and maintenance. The miniaturization of explants allows reduction in space requirements and consequently labor cost for the maintenance of germplasm collection. There are two types of *in vitro* conservation of plant germplasm. First is the slow growth which achieved by modifying the culture

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medium or reducing temperature requirements (Withers, 1991). Second is cryopreservation which understood as storage between -79 and -196 ˚C, the low extreme being the temperature of liquid nitrogen. In this respect, date palm germplasm have been preserved *in vitro* in the form of shoot tips, callus cultures and somatic embryos (Tisserat, 1981; Mater, 1987; Bekheet *et al*., 2001; Bekheet *et al*., 2005). Moreover, biochemical and molecular markers have successfully used for detection of genetic variation of date palm tissue cultures (Saker *et al*., 2000; Bekheet *et al.,* 2007; Othmani *et al*., 2009). The purpose of this study is to investigate the effect of low temperature and darkness conditions on *in vitro* storage of embryogenic cultures of two dry cultivars i.e., Bartamoda and Sakkoty of Egyptian date palm.

Materials and Methods

1- Establishment of embryogenic cultures:

Offshoots of female date palm cvs. Bertamoda and Sakkoty were used as plant materials. Leaves were gradually removed and shoot apices 5 cm in length were taken and kept in antioxidant solution (100 mg/l ascorbic acid + 150 mg/l citric acid). Explants were sterilized using 70 % ethanol for 1 min. and 2.6 % sodium hypochlorite for 20 min. then rinsed three times with sterile distilled water. Shoot tips trimmed to about 1 cm were excised with part of submeristematic tissues and aseptically cultured on (MS) medium (Murashige and Skoog 1962) supplemented with 2 mg/l dimethyl amino- purine (2 iP) + 1 mg/ l naphthalene acetic acid (NAA). Cultures were then incubated in darkness and recultured on the same fresh medium every four weeks. For induction of embryogenic callus, cultures were transferred onto MS medium supplemented with 10 mg/ l 2, 4-dichlorophenoxy acetic acid (2,4- D) + 3 mg/l 2iP according to Bekheet *et al*. (2005).

2- Low temperature storage:

In this experiment, clusters of embryogenic cultures were transferred into jars (80 × 40 mm) containing embryogenic callus medium described previously, and incubated at 5^{o}C in a refrigerator in darkness, in parallel with embryogenic cultures incubated in a growth chamber at 24 ± 2^{o}C in a complete darkness as a control. Percentage of survival and healthy cultures were recorded after 3, 6, 9 and 12 months of storage using ten replicates. The number of proliferated embryos was recorded and the browning degree of the tissues was investigated.

3- Storage at different illumination conditions:

To study the effect of storage at darkness condition, equal pieces of embryogenic cultures of the two date palm cultivars were transferred into jars (80 × 40 mm) containing embryogenic callus medium and then incubated at room temperature (24± 2 º C) in complete dark conditions. Others cultures were incubated in a growth chamber at 24 ±2 ºC under light conditions of 16 hr. photoperiod at intensity of 2000 lux from cool white fluorescent lamps (control). Percentage of survival and healthy cultures were recorded after 3, 6, 9 and 12 months of storage using ten replicates. The number of proliferated embryos was recorded and the browning degree of tissues was investigated.

4- Culture conditions:

The media used were contained 30 g/l sucrose and 7 g/l agar and they were adjusted at PH 5.8 using 0.1 N of either KOH or HCL, and autoclaved at a pressure of 1.2 Kg cm2 for 24 min. The normal incubation conditions were: temperature of 25 ± 2^{o}C photoperiod 16 hr, irradiance of 45 µmol m^{-2} s^{-1} (Philips white fluorescent tubes).

5- Isolation of genomic DNA and RAPD analysis:

DNA was isolated using the Cetyl Trimethyl Ammonium Bromide (CTAB) method of Doyle and Doyle (1990). RAPD analysis was carried out using five oligonucleotide primer (9 - 10 mer) A11 (5/- TGGCGACCTG -3/), A12 (5/- GAGGCGTCGG -3/) , A6 (5/ CCCTACCGAC -3/), A10(5/ TCGTTCCGC -3/) and A13 (5/ CACCTTTCCC -3/) to detect the polymorphism among the plantlets derived from two different preservation methods in present of *in vitro* cultures as a control. The amplification was carried out in 25 µl reaction volume containing DNA master mix 12.5 µl (PCR buffer, $Mgcl_2$, dNTps, Taq DNA polymerase), primer 2 µl, template DNA 2 µl and sterilized distilled water 8.5 µl. PCR. Amplification was performed for 40 cycles, using UNO thermalcycler of Biometra (Germany) as follows: one cycle at 92 °C for 2 min then 40 cycles at 94°C for 30 s, 36°C for 1 min and 72°C for 30 sec (for denaturation, annealing and extension, respectively). Reaction mixture

was finally incubated at 72°C for 10 min. Change into: The amplification products were analyzed by electrophoresis in 1% agarose in TBE (Tris-Borate-EDTA) buffer (pH 8.0) in presence of 100 bp DNA ladder (Promega) was used as a marker with a molecular size of 3000, 1500, 1000, 900, 800, 700, 600, 500, 400, 300, 200 and 100 bp ,then stained with ethidium bromide (0.2 mg /ml) and photographed under UV light.

6- Statistical analysis:

Experiments were run in completely randomized design and data were statistically analyzed using SAS program according to the method described by Snedecor and Cochran (1967).

Results and Discussion

1- Low temperature storage:

Results of Table (1) showed that the highest percentage for survival (100 %) recorded in both Bartamoda and Sakkoty cultivars after three months of cold preservation (5 ºC) in comparison to normal temperatures for the same period (65.50 % and 78.25 %, respectively). Survival percentages were decreased as storage period increased in both cultivars. After twelve months Sakkoty registered 88. 88 % of survival and 87.7 % was observed with Bartamoda cultivar with considerable browning (Table 2).

In concern of embryogenic cultures proliferation, results tabulated in Table (3) and illustrated in Fig. (1) revealed that cold storage at 5ºC gave lower rate of embryos proliferation comparing with cultures stored at normal temperatures. Bartamoda gave 2.40 embryos /cluster after 12 months of cold storage while, 2.33 embryos /cluster was obtained with Sakkoty cultivar. However, 4.03 and 4.92 embryos/cluster was proliferated by Bartamoda and Sakkoty, respectively at normal temperatures storage.

Table 1: Effect of low temperature (5 °C) on percentages of mortality and survival of embryogenic cultures of the date palm cultivars i.e. Bartamoda and Sakkoty during 12 months of culturing at darkness.

Incubation conditions	Time (months)	Mortality %			Survival %		
		Bartamoda	Sakkoty	Means	Bartamoda	Sakkoty	Means
Normal temperature (control)	3	34.50 C	21.75 D	28.12 B	65.50 CD	78.25 B	71.85B
	6	47.70 B	27.80 D	37.80 B	52.30 D	72.20BC	62.25 B
	9	51.22 A	30.77 CD	40.99 A	48.88 DE	69,23BC	59.01 C
	12	55.14 A	33.15 C	44.15 A	44.86 E	66.85CD	55.85 C
Low temperature storage	3	0.00F	0.00F	0.00 C	100.00 A	100.00 A	100.00A
	6	0.00F	6.66 F	3.33 C	100.00 A	93.33AB	96.67 A
	9	1.11F	10.00 E	5.56 C	98.88 A	90.00AB	94.44 A
	12	12.22DE	11.11 DE	11.67 C	87.77 AB	88.88AB	88.33 A
Means		25.23 A	13.80 B		74.77 B	82.34 A	

Means with the same letters are not significantly different

Table 2: Effect of low temperature (5°c) storage on browning degree of embryogenic cultures of the date palm cultivars i.e. Bartamoda and Sakkoy during 12 months of culturing at darkness.

Incubation conditions	Time (month)	Browning	
		Bartamoda	Sakkoty
Normal temperature (control)	3	-	-
	6	+	-
	9	++	+
	12	+ +	++
Low temperature storage	3	-	-
	6	-	-
	9	+	+
	12	++	+

+ = slightly brown ++= brown +++= dark brown - = white -- = green --- = dark green

Storage at low temperature is one of the major tissue culture techniques used for preservation of plant resources since growth reduction is generally achieved by lowering the incubation temperature. Under such condition, accumulation of unsaturated lipids on the cell membrane would cause cell membrane thickening and retard cell division and elongation (Engelmann, 1991 and 1997).

The previous results revealed that date palm embryogenic cultures remained healthy without any serious signs of senescence during the different storage periods. Slight browning of the preserved cultures was then noticed after 12 months of storage. The present results are in accordance with those reported by Bekheet *et al*., (2001). They mentioned that shoot buds and callus cultures of date palm cv. Zaghlool were successfully stored for 12 months at 5 ºC in dark. On the other hand, 80% of pear and 85% of apple shoot bud cultures grown *in*

vitro remained alive after eighteen months storage at 4ºC and 8ºC, respectively (Wannas *et al*., 1986 and Wannas, 1992). Otherwise, Hao and Deng (2003) reported that, shoot-tips of apple cultivar 'Gala' were stored *in vitro* using a low temperature slow-growth culture method. All shoot-tips survived 1-year storage, with a significant height increment over that period. On the contrary, Corbineau *et al*. (1990) mentioned that oil palm plantlets and somatic embryos were not able to resist a relatively short exposure to temperatures lower than 18 ºC.

Table 3: Effect of low temperature (5 ° C) storage on number of embryos / culture of the two date palm cultivars i.e. Bartamoda and Sakkoty during 12 months of culturing at darkness.

Incubation conditions	Time (month)	No. embryos / culture		
		Bartamoda	Sakkoty	Means
Normal temperature (control)	3	1.88 C	2.00 C	1.94 B
	6	2.33 BC	2.89 B	2.61 AB
	9	3.06 AB	3.52 A	3.29 A
	12	4.03 A	4.92 A	4.47 A
Low temperature storage	3	0.77 D	0.44 D	0.61 C
	6	1.44 C	1.33 C	1.39 C
	9	2.33 BC	2.22BC	2.25BC
	12	2.40 BC	2.33 BC	2.35BC
Means		2.28 A	2.45 A	

Means with the same letters are not significantly different.

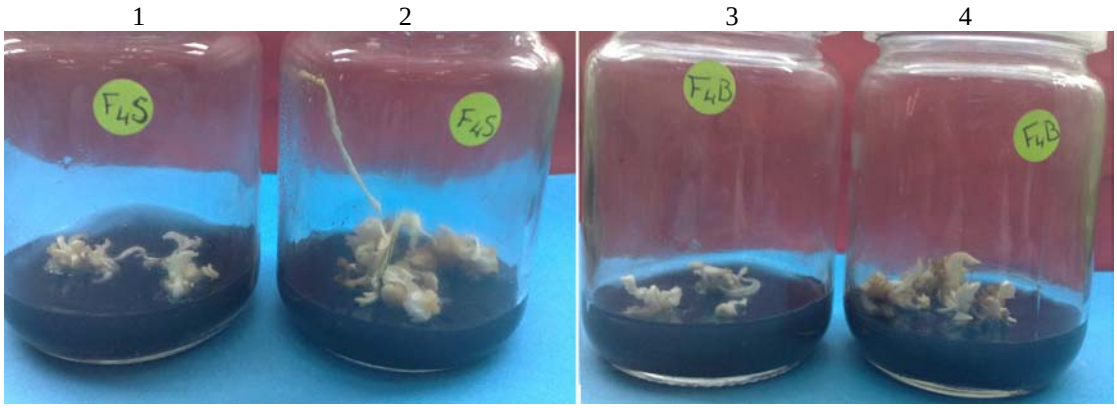

Fig. 1: Embryogenic cultures of date palm cvs. Sakkoty and Bertamoda stored for twelve months 1: Sakkoty at 5 ° C, 2: Sakkoty at normal temperature, 3: Bartamoda at 5 ° C and 4 : Bartamoda at normal temperature all cultures were kept at darkness.

2- Storage at two illumination conditions:

Table (4) showed the survival and mortality percentage of embryogenic cultures of Bartamoda and Sakkoty cvs. stored at darkness during twelve months: and cultures of previous cultivars were incubated at two illumination conditions i.e. light and darkness. Results cleared that survival percentage was declined as the storage period increased in both cultures incubated in light and dark conditions for the two cultivars. After the twelfth month of storage, the survival percentages were declined roughly in both two conditions of storage. It was noticed that storage in darkness conditions recorded high survival percentages for the two cultivars in comparison to storage at light conditions (at darkness, Bartamoda recorded 73.73, 61.82 and 58.56 after 3, 6 and 9 months, respectively). While at light conditions, Bartamoda recorded 61.11, 38.89 and 28.89 after 3, 6 and 9 months, respectively. For explants browning during storage period, data tabulated in Table (5) and illustrated in Fig. (2) cleared that storage in darkness for six months caused whiteness of explants. After the sixth month, explants changed into brownnosing. While, those were kept in light conditions until the ninth month kept green. The proliferation of embryogenic cultures, storage in light conditions of the two cultivars caused increase in embryo proliferation rate within increasing storage period only till the ninth month (Table 6). On the other hand, storage in darkness conditions Bartamoda registered increasing in embryogenic proliferation rate until the sixth month then non-significant increasing while Sakkoty gave increasing number of embryos / culture to the twelfth month with significant value but less than that kept in light conditions.

Table 4: Effect of illumination conditions (light and darkness) on percentage of mortality and survival of embryogenic cultures of the two date palm cultivars i.e. Bartamoda and Sakkoty during 12 months of culturing:

Incubation conditions	Time (month)	Mortality %			Survival %		
		Bartamoda	Sakkoty	Means	Bartamoda	Sakkoty	Means
Light	3	38.89 C	25.56 D	32.23 B	61.11 C	74.44 AB	67.78AB
	6	61.11 AB	36.67 C	48.89 AB	38.89 D	63.33 BC	51.13B
	9	71.11A	42.22BC	56.67 A	28.89 E	57.78 C	43.34C
	12	72.22 A	46.67BC	59.45 A	27.78 E	53.33 C	40.56C
Darkness	3	26.27 D	15.80 E	21.03 C	73.73 AB	84.20 A	78.97A
	6	38.18 C	25.28 D	31.73 B	61.82 C	74.72 AB	68.27 A
	9	41.44 BC	29.12CD	35.28 B	58.56 C	70.88 AB	64.72AB
	12	61.10 AB	48.88 BC	54.99 A	38.90 D	51.12 C	45.01C
Means		51.29 A	33.77 B		48.71B	66.22A	

Means with the same letters are not significantly different.

Table 5: Effect of illumination conditions (light and darkness) on browning degree of embryogenic cultures of the two date palm cultivars i.e. Bartamoda and Sakkoy during 12 months of culturing:

Incubation conditions	Time (month)	Browning	
		Bartamoda	Sakkoty
Light	3	--	--
	6	--	--
	9	++	++
	12	+++	++
Darkness	3	-	-
	6	-	-
	9	+	+
	12	++	++

+ = slightly brown ++= brown +++= dark brown brown - = white -- = green --- = dark green

Table 6: Effect of illumination condition (light and darkness) on number of embryos / culture of the two date palm cultivars i.e. Bartamoda and Sakkoty during 12 months of culuring.

Incubation conditions	Time (month)	No. embryos / culture		
		Bartamoda	Sakkoty	Means
Light	3	3.00 D	3.16 D	3.08 C
	6	5.40 C	6.33 B	5.87 B
	9	7.60 AB	8.50 A	8.05 A
	12	7.70 AB	8.56 A	8.13 A
Darkness	3	1.56 E	1.89 E	1.72 D
	6	1.99 E	2.73 E	2. 36 D
	9	2.97 DE	3.30 D	3.13 C
	12	3.87 D	4.50 C	4.18 BC
Means		4.26 B	4.87 A	

Means with the same letters are not significantly different.

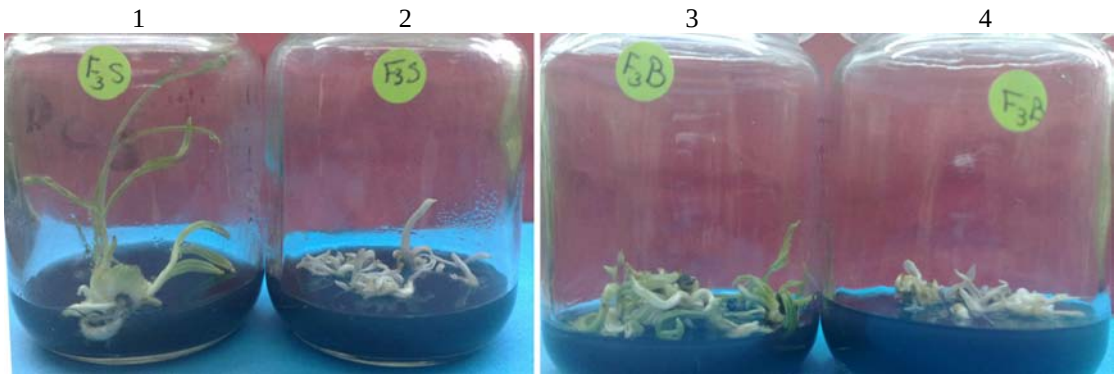

Fig. 2: Embryogenic cultures of date palm cvs. Sakkoty and Bartamoda stored for twelve months 1: Sakkoty stored in dark, 2: Sakkoty stored in light conditions, 3: Bartamoda stored in dark and 4: Bartamoda stored in light conditions.

Generally, our results revealed that embryogenic culture of date palm cvs. Bartamoda and Sakkoty stored at 5 ºC in complete darkness remained viable with considerable survival percentage for twelve month. However, using darkness at room temperature was effective only for six months. These results are in accordance with those reported by Bekheet *et al*. (2001) in their study on date palm shoot buds and callus cultures, they mentioned that shoot buds and callus cultures were successfully stored for 12 months at 5 ºC in dark. In this respect, Read and Preece (2003) reported that plant photosynthesis processes rely on an interplay of light and dark reactions in order to produce the materials needed for growth. The effects of light enable plants to convert the energy contained in light into a chemical process. During dark periods, plants convert the energy contained inside these chemical processes into glucose, the material that fuels plant growth activities.

3- Molecular analysis:

RAPD analysis was used to determine the genetic stability of treated and non-treated tissue cultures of date palm and testing the similarity of both cultures. Five randomly selected primers were used. Two of them (A6 and A11) did not give reproducible and sufficient amplification products. DNA fragments varied in numbers and sizes depending on the primers used and the different cultivars.

Data presented in Table (7) and shown in Fig (3 and 4) indicate the RAPD analysis for *in vitro* preservation treatments of the two date palm cultivars using three primers, A10, A12 and A13 revealed that the three primers used produced 21 bands with nine polymorphic bands for Bartamoda cultivar. All three primers gave the same number of polymorphic bands. The percentage of polymorphism recorded for all primers was 42.85. However for Sakkoty cultivar, the same primers used produced 16 bands with eleven polymorphic bands, the largest number of amplified bands were recorded with primer A13 (7 bands) while, the lowest number of amplified bands were observed with primer A10 (4 bands). However, the percentage of polymorphism recorded for primer A13 was 85.71 as the highest value whereas, the percentage recorded with primer A10 was 50 as the lowest value. It was clear, the over all total polymorphism for Bartamoda (42.85) was lower than polymorphism for Sakkoty (68.75).

Table 7: Total number of bands, polymorphic bands and percentage of polymorphism as revealed by RAPD markers among the different *in vitro* preservation treatments of the two date palm cultivars i.e. Bartamoda and Sakkoty:

cultivars	Primer code	Sequence 5′--------3′	Total no. of bands	Polymorphic bands	Polymorphism %
Bartamoda	A10	TCGTTCCGC	7	3	42.85
	A12	GAGGCGTCGG	7	3	42.85
	A13	CACCTTTCCC	7	3	42.85
Over all total			21	9	42.85
Sakkoty	A10	TCGTTCCGC	4	2	50.00
	A12	GAGGCGTCGG	5	3	60.00
	A13	CACCTTTCCC	7	6	85.71
Over all total			16	11	68.75

T,C,G and A refer to Thyamidine, Cytodine, Guanidine and Adenine, respectively.

Table 8: Distribution and size of polymorphic bands among the different *in vitro* preservation treatments of the two date palm cultivars i.e. Bartamoda and Sakkoy using three primers.

Cultivar	primer	Polymorphic band	Cold Temp.	Normal Temp.	Light	dark
Bartamoda	A10	1000	-	+	-	+
		800	-	+	-	+
		600	-	+	-	+
	A12	1300	-	+	-	+
		1000	+	+	+	-
		800	-	+	-	+
	A13	1000	-	+	-	+
		800	-	+	-	+
		600	-	+	-	+
Sakkoty	A10	270	-	+	+	+
		172	+	+	-	+
	A12	800	-	-	+	-
		700	+	-	+	-
		600	-	-	+	-
	A13	800	_	+	-	+
		700	-	+	-	+
		600	-	+	-	+
		450	-	+	-	+
		400	-	+	+	+
		300	-	+	+	+

+ and - indicate the presence and absence of polymorphic bands, respectively.

The presence and absence of polymorphic bands among *in vitro* preservation treatments of the two date palm cultivars shown in Table (8). For Bartamoda, the three primers used, produced nine amplification products that were monomorphic among all treatments ranged from 600 to 1300 bp. Whereas for Sakkoty, three primers gave eleven amplification products that were monomorphic among all treatments ranged from 170 to 800 bp.

From the obtained results, it could be concluded that at DNA molecular level, RAPD analysis of *in vitro* preservation treatments of the two date palm cultivars exhibited a genetic variations. The Bartamoda cultivar gave the highest percent of similarity (57.5) and Sakotty cultivar gave the lowest percent of similarity (31.25).This variation is due to the somaclonal variations occurred during culturing of explants in non-normal conditions and proliferation of callus tissues with high variation in cell division or differentiation under *in vitro* conditions.

The present results are in contrast with those reported with Bekheet *et al* .(2007) and with those reported by Saker *et al. (*2000), they mentioned that no significant variation observed of tissue cultures derived plantlets. RAPD analysis showed genetic variation in only 4 % of analyzed plants (70 regenerants) which were incubated for 6- 12 months under 25 ^{O} C.

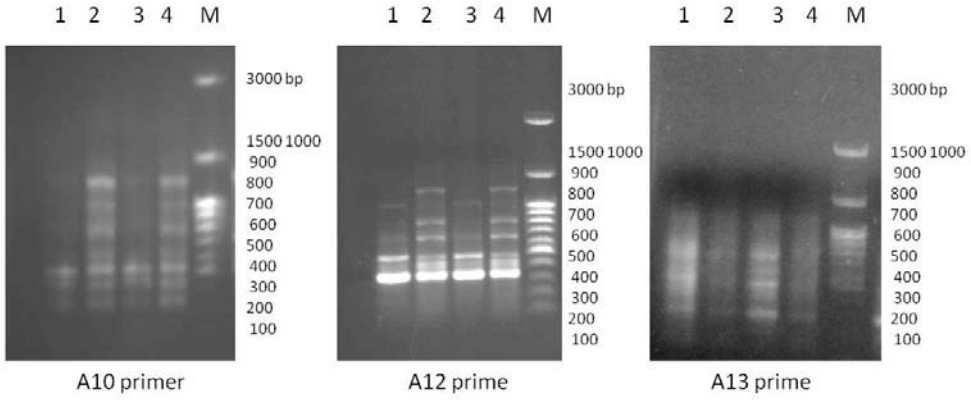

Fig. 3: Agarose gel electrophoresis of randomly primed DNA amplifications of different *in vitro* preservation treatments of Bartamoda cultivar.

M refers to the DNA marker given in bp.
Lane 1 indicates preservation in cold temperature.
Lane 2 indicates preservation at normal temperature.
Lane 3 indicates preservation in light.
Lane 4 indicates preservation in darkness.

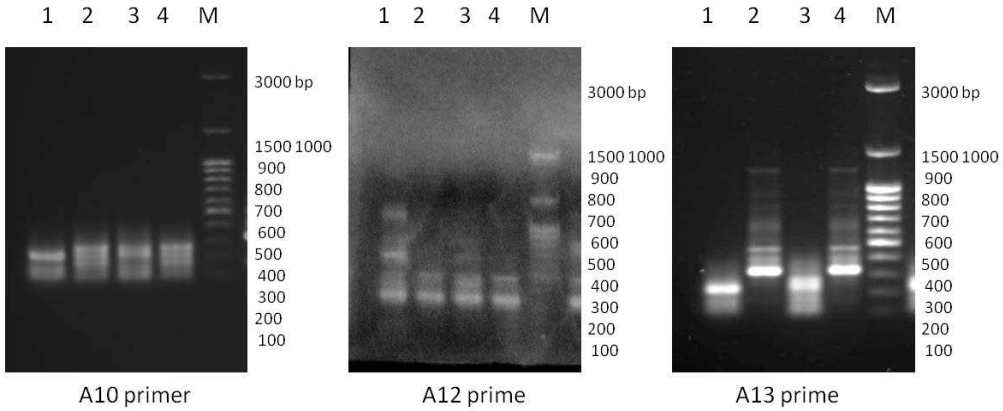

Fig. 4: Agarose gel electrophoresis of randomly primed DNA amplifications of different *in vitro* preservation treatments of Sakkoty cultivar.

M refers to the DNA marker given in bp.
Lane 1 indicates preservation in cold temperature.
Lane 2 indicates preservation at normal temperature.
Lane 3 indicates preservation in light.
Lane 4 indicates preservation in darkness.

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